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"Pulmonary and systemic endothelial function in diabetic animal models: effect of carotid sinus nerve denervation"

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Resumo

As doenças metabólicas afetam milhares de indivíduos em todo o mundo e representam um grupo de doenças crónicas de elevada prevalência. A diabetes mellitus tipo 2 (DMT2) é um dos maiores problemas mundiais que afetou, em 2015, aproximadamente 415 milhões de pessoas e acredita-se que a sua prevalência vai continuar a aumentar nos próximos anos. Esta patologia é uma condição metabólica crónica, caracterizada pela incapacidade de os tecidos periféricos usarem a insulina, produzida pelas células β, resultando numa condição designada de resistência à insulina, o que leva a hiperinsulinemia e consequentemente a hiperglicemia. Esta é a principal característica da DMT2 e é também um fator de risco para o desenvolvimento de doenças cardiovasculares, como a hipertensão e aterosclerose, sendo fulcral na disfunção endotelial. As complicações vasculares da diabetes dividem-se em microvasculares e macrovasculares.

O óxido nítrico (NO) é um potente vasodilatador e tem sido identificado pela sua participação na regulação do tónus vascular, fluxo sanguíneo e na modelação da libertação de mediadores do endotélio. O NO é produzido pelo NO endotelial juntamente com o cofator tetrahidrobiopterina, pela conversão da L-arginina em L-citrulina, nas células endoteliais, num processo regulado pelo stress, condições de oxigénio e inflamação. Foram identificadas três isoformas do NO sintase: NO neuronal sintase (nNOS), NO endotelial sintase (eNOS) e NO induzível sintase (iNOS).

A hipertensão sistémica é um dos principais fatores de risco para as doenças cardiovasculares e contribui para uma elevada resistência vascular periférica. Demonstrou-se que em animais hipertensos a vasodilatação está reduzida e que essa diminuição pode estar relacionada com a redução da disponibilidade de óxido nítrico (NO). Também a hipertensão arterial pulmonar tem vindo a ser cada vez mais associada à DMT2 e à disfunção endotelial, e esta relação tem sido descrita como estando relacionada com o aumento da produção de superóxido, que por sua vez reage com o NO, reduzindo a sua difusão, o que leva a um aumento da vasoconstrição. A prostaglandina $F_2\alpha$ ($PGF_2\alpha$) é um dos mais frequentes prostanoides estudados pela sua ação contráctil no musculo liso. O recetor FP é um recetor específico da $PGF_2\alpha$, que está associado a diferentes condições fisiopatológicas como a resistência à insulina, problemas cardiovasculares e diabetes.

A disfunção endotelial é definida como um desequilíbrio entre a vasodilatação e a vasoconstrição do endotélio, por ação das substâncias produzidas neste. Uma das características usadas para detetar se há complicações na função endotelial, é investigar se a resposta fisiológica aos vasodilatadores está comprometida. Para tal é usualmente utlizada a resposta vasodilatadora à acetilcolina (ACh), que medeia a vasodilatação através do NO, produzido pelo endotélio. Na literatura, também é igualmente aceite que a disfunção endotelial está associada à diminuição da disponibilidade de NO. Esta diminuição pode ser causada pela redução na produção de NO pelo endotélio ou pelo aumento da sua inativação, pelas espécies reativas de oxigénio.

O eNOS mantém várias funções homeostáticas nos vasos sanguíneos, como o relaxamento do endotélio, no entanto uma diminuição no eNOS pode levar ao desenvolvimento de resistência à insulina e problemas metabólicos, que são complicações que estão na génese de patologias, como a diabetes e hipertensão. Por outro lado, a expressão da iNOS é ativada por patologias de doenças inflamatórias, incluído a diabetes e a aterosclerose, sendo esta proteína identificada como essencial na resistência à insulina, na obesidade e nas disfunções contrácteis cardíacas e vasculares. O NO foi proposto como sendo um modulador inibitório do corpo carotídeo (CB).

Os CBs são quimiorrecetores periféricos que detetam alterações no oxigénio, dióxido de carbono e níveis de pH no sangue arterial. Sabe-se que a sua atividade está aumentada em modelos de roedores com resistência à insulina e o nosso grupo mostrou anteriormente, que a resseção bilateral do nervo do seio carotídeo (CSN), previne por completo a resistência à insulina, hiperglicemia, dislipidemia e hipertensão arterial.

Assim, dado que a disfunção endotelial pode resultar ou contribuir para o desenvolvimento de várias doenças como a hipertensão e DMT2 e sabendo que a hiperativação do CB está associada a estas doenças, o principal objetivo deste trabalho foi avaliar o impacto da resseção do CSN, na função endotelial da artéria pulmonar e no sistema vascular sistémico, como a artéria aorta.

As experiências foram realizadas em ratos Wistar machos com 3 meses de idade. Estes animais foram submetidos a uma dieta hipercalórica (HFHSu) (60% de gordura) juntamente com 35% de sacarose na água durante 25 semanas. Este modelo é considerado uma fase inicial da DMT2, desenvolvendo assim resistência à insulina, intolerância à glucose e hipertensão. Estes animais foram comparados com um grupo controlo da mesma idade submetidos a uma dieta padrão. Após 14 semanas de dieta HFHSu, foi confirmada a resistência à insulina através dos testes de sensibilidade à insulina e de tolerância à glucose. Em seguida, os animais foram divididos aleatoriamente, em que metade foram submetidos a uma ressecção bilateral do CSN, e a outra metade submetidos ao mesmo procedimento cirúrgico, mas sem corte do CSN (grupo *sham*). Depois da cirurgia, os animais foram mantidos nas respetivas dietas e passado duas e onze semanas após a resseção do nervo, a sensibilidade à insulina e tolerância à glucose foram avaliadas.

Passado este período, os animais foram submetidos a uma experiência terminal, onde os animais foram anestesiados com pentobarbital (60mg/kg, i.p.). O sangue foi recolhido por punção cardíaca para medir os níveis de NO por quimiluminescência. A artéria pulmonar e a aorta foram excisadas e dissecadas para estudos da função vascular, através de miografia, e para posterior homogeneização e análise da expressão, por Western Blot, e quantificação de NO.

A sensibilidade à insulina foi avaliada pelo teste da tolerância à insulina (ITT), a tolerância à glucose pelo teste da tolerância à glucose oral e a pressão arterial foi registada através do transdutor e amplificador de pressão. Para além disso a função endotelial foi avaliada através da miografia, sendo as respostas contráteis com estímulo ao K^+ e prostaglandina $F_2\alpha$ ($PGF_2\alpha$) e as respostas vasodilatadoras induzidas pela ACh. Os níveis de NO no plasma, artéria pulmonar e aorta foram medidos através quimiluminescência e por fim a expressão das isoformas do NO sintase: eNOS e iNOS e do recetor da $PGF_2\alpha$ (FP) foram analisados por Western Blot.

Observou-se que a dieta HFHSu diminui a sensibilidade à insulina e aumentou a intolerância à glucose e a pressão arterial. A dieta hipercalórica aumentou os níveis de NO no plasma e as repostas contrácteis à $PGF_2\alpha$ na aorta. Aumentou ainda os valores da expressão, tanto do iNOS, em ambas as artérias, como do recetor FP, na artéria pulmonar. No entanto, a dieta HFHSu, diminui a resposta contráctil à $PGF_2\alpha$ na artéria pulmonar, o relaxamento à ACh em ambas as artérias e o nível de NO na aorta. Por outro lado, a resseção do CSN, restaurou para valores normais a sensibilidade à insulina, a tolerância à glucose e a pressão arterial. A resseção do CSN ainda normalizou os valores do NO no plasma e diminui as repostas ao K^+ na artéria pulmonar e na aorta. Por fim, ainda restaurou por completo a vasodilatação dos animais HFHSu e normalizou os valores de expressão da iNOS, em ambas as artérias, e restabeleceu os níveis de expressão do recetor FP na artéria pulmonar. Os níveis de expressão do eNOS, em ambas as artérias, não sofreram nenhuma modificação nem pela dieta nem pela resseção do CSN.

Neste estudo foi demonstrado pela primeira vez que a resseção do CSN nos animais HFHSu restaurou a função endotelial na artéria pulmonar e na aorta. Verificou-se também que a resseção do CSN nos animais HFHSu diminuiu os níveis de NO no plasma e que normalizou os valores da expressão do iNOS na artéria pulmonar e na aorta, bem como os valores da expressão do recetor FP na artéria pulmonar.

Palavras-Chaves: corpo carotídeo, diabetes tipo 2, disfunção endotelial, nervo do seio carotídeo, óxido nítrico

Abstract

Diabetes mellitus type 2 (DT2M) is a metabolic disease characterized by the inability of peripheral tissues to use insulin in the target organs, resulting in a condition known as insulin resistance, which leads to elevated levels of insulin and consequently glucose in blood.

Systemic hypertension and arterial pulmonary hypertension have been associated, over the years, with DT2M and endothelial dysfunction, and it has been shown that hypertensive animals, exhibit a reduction in the vasodilation which in turn is related to a diminished nitric oxide (NO) levels, leading to a vasoconstriction in the vessels. Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) is a powerful vasoconstrictor which have been implicated in several pathological states. $PGF_2\alpha$ cause vasoconstriction primarily through the FP receptor.

There are numerous mechanisms that could cause endothelial dysfunction, but the most consensual is the decreased bioavailability of NO levels. NO is a vasodilator that helps to regulate the vascular tone and blood flow. It is produced by the endothelial NO with the cofactor tetrahydrobiopterin, through the conversion of L-arginine in L-citrulline. NO has been proposed to be an inhibitory modulator of the carotid body (CB) chemosensory process.

The CBs are peripheral chemoreceptors that are known to be involved in several cardiometabolic pathologies, as essential hypertension, chronic heart failure and metabolic syndrome. It has been recently described that CBs activity is raised in models of insulin resistance and that carotid sinus nerve (CSN) resection can restore insulin resistance, hyperglycaemia, dyslipidaemia and arterial hypertension.

Therefore, the main goal of this project was to evaluate the impact of CSN resection on the endothelial dysfunction in pulmonary artery and systemic vessels. Experiments were performed in 3 months old Wistar rats, fed with a high-fat and high-sucrose diet (HFHSu) during 25 weeks, or fed with a standard diet (CTL) during the same period. To evaluate the impact of CSN denervation, the animals were submitted to CSN resection at the fourteen weeks of diet. Insulin sensitivity, glucose tolerance, mean blood pressure, endothelial function in pulmonary artery and aorta, plasmatic NO levels and the expression of NO synthase isoforms in the pulmonary artery and aorta: endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) and also $PGF_2\alpha$ receptor (FP) were evaluated in CTL and HFHSu animal models.

Herein, HFHSu diet modify all the parameters above describe, except the contractile responses to K^+ in both arteries, the NO levels in the pulmonary artery and the levels of expression of FP receptor on aorta. On the other hand, the CSN resection almost completely restored all the changes caused by the HFHSu diet-induced, apart from the NO levels in both arteries and the expression levels of FP receptor in the aorta. eNOS expression levels in both arteries were not modified nor by the diet neither by CSN resection.

We demonstrated for the first time, that CSN resection restores completely endothelial function in the pulmonary artery and in the aorta. It was also shown that CSN resection decreased NO levels in plasma and normalized the levels the expression of iNOS, in both arteries, as well as the levels of expression of the FP receptor in the pulmonary artery.

Key-words: Carotid body, diabetes type 2, endothelial dysfunction, carotid sinus nerve, nitric oxide

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Abbreviation list

ACh – Acetylcholine
ADMA – Asymmetric Dimethyl Arginine
AUC – Area under the Curve
BH4 – Tetrahydrobiopterin
CB – Carotid Body
cGMP – Cyclic Guanosine Monophosphate
CRC – Concentration–Response Curve
CSN – Carotid Sinus Nerve
CTL – Control
DM – Diabetes Mellitus
EDCF – Endothelium-derived Contracting Factor
EDRF – Endothelium-derived Relaxing Factor
ET-1 – Endothelin-1
eNOS – Endothelial Nitric Oxide Synthase
GTP – Guanosine-5'-Triphosphate
iNOS – Inducible Nitric Oxide Synthase
IR – Insulin Resistant
ITT – Insulin Tolerance Test
K_{ITT} – Constant Rate for Plasma Glucose Decline
KPSS – Physiological Salt Solution
HFHSu – High-fat/ High-sucrose
MAPK – Mitogen Activated Protein Kinase
MBP – Mean Blood Pressure
NADPH – Nicotinamide Adenine Dinucleotide Phosphate
nNOS – Neuronal Nitric Oxide Synthase
NO – Nitric Oxide
OGTT – Oral Glucose Tolerance Test
O_2^- – Superoxide
$ONOO^-$ – Peroxynitrite Anion
PAH – Pulmonary Arterial Hypertension
$PGF_2\alpha$ – Prostaglandin F2 α
PSS – Krebs-Henseleit buffer
FP receptor – Prostaglandin F2 α receptor (FP)
PI3K – Phosphatidylinositol 3 Kinase
ROS – Reactive Oxygen Species
SDS-PAGE – Dodecylsulfate-Polyacrylamide Gel Electrophoresis
S1177 – Serine 1177
SNS – Sympathetic Nervous System
T2DM – Type 2 Diabetes Mellitus
TBST – Tris-buffered Saline with Tween 20
TNF-α – Tumor Necrosis Factor-α
VSMC – Vascular Smooth Muscle Cell

1. Introduction

1.1 Metabolic syndrome and diabetes mellitus

Metabolic syndrome is a defined cluster of metabolic abnormalities (including visceral obesity, dyslipidaemia, hypertension and impairment of glucose metabolism) that rise the risk for developing type 2 diabetes mellitus (T2DM), cardiovascular disease and coronary heart disease [1]. This type of syndrome is becoming more common due to an increase of risk factors including alcohol abuse, insufficient physical activity, aging, stress and a sugar rich diet [2].

Diabetes mellitus (DM) is a widespread metabolic disorder that is characterized by insufficient production of insulin or the incapacity to use the insulin that the β-cells produce [3]. Insulin is a hormone produced in the pancreatic β-cells that acts on its target tissues (mainly liver, muscle and fat) in response to glucose ingestion [4]. Insulin stimulates the glucose transport from the bloodstream through the cell membrane of the target tissues with the cooperation of glucose transport proteins in order to maintain low glucose levels in blood [4]. The ineffectiveness of insulin to stimulate glucose uptake, leads to an increased concentration of glucose in the blood (hyperglycaemia) [5].

People with diabetes have a higher risk for developing several complications, including, cardiovascular disease, blindness, kidney failure and lower limb amputation due to the high levels of glucose in blood [5].

According to International Diabetes Federation, DM affected in 2015 at least 415 million people worldwide and it is expected to reach 642 million people by the year 2040 (see Figure 1.1). In the Portuguese population, it is estimated that the prevalence of diabetes in adults between the ages of 20-79 is 13.6%. It was assessed that 7896 deaths was related with diabetes [6].

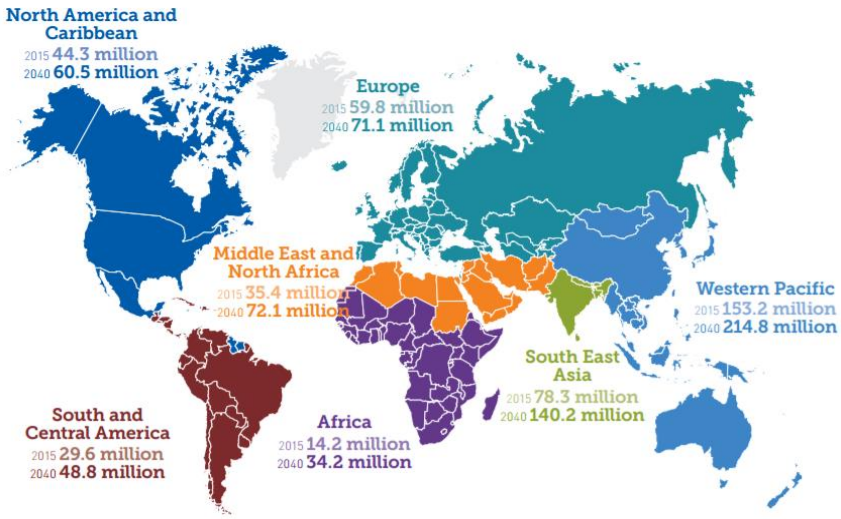

Figure 1.1- Estimated number of people with diabetes mellitus worldwide per region in 2015 and 2040 [6].

There are three predominant types of diabetes such as type 1 diabetes, that results from an autoimmune pancreatic β-cells destruction which leads to absolute insulin deficiency; type 2 diabetes, characterized by insulin resistance (IR) and relative insulin deficiency; and gestational diabetes, defined as any slight degree of glucose intolerance during the pregnancy [4].

1.1.1 Type 2 Diabetes Mellitus

T2DM is the most prevalent type of DM and over the past few years had increased significantly. There are several risk factors that influence the emerging of T2DM such as excess body weight, poor nutrition, physical inactivity, genetics, older age and family history of diabetes [6].

This type of diabetes is characterized by the inability to use insulin efficiently in the target organs and an inadequate compensatory insulin secretory response to elevated glucose concentration [4], resulting in a condition known as IR [7]. T2DM evolved from an asymptomatic stage with IR, to a slight postprandial hyperglycaemia. The chronic hyperglycaemia plays a major role in the initiation of diabetes with microvascular (including retinopathy, nephropathy and neuropathy) and macrovascular (including ischemic heart disease, peripheral vascular disease and cerebrovascular disease) complications (figure 1.2) [8].

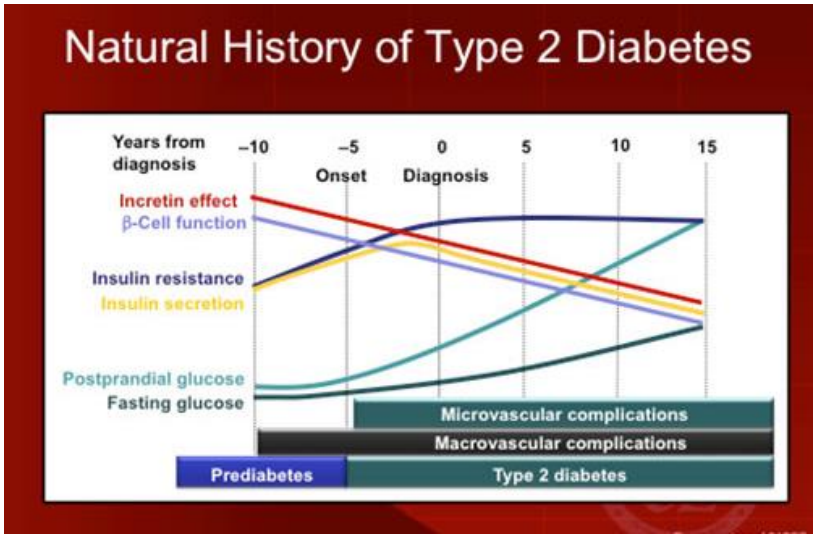

Figure 1.2- Natural history of type 2 diabetes, since the progressive impaired glucose tolerance to overt type 2 diabetes. Initially the β-cell manage to compensate insulin resistance by increasing the insulin secretion, which results in hyperinsulinemia. Over time the impaired glucose tolerance progresses to mild postprandial hyperglycaemia. When β-cell begins to fail, insulin deficiency, fasting hyperglycaemia and type 2 diabetes with microvascular and macrovascular complications develops [9].

1.2 Insulin Resistance

IR is a pathological condition in which the ability of peripheral tissues to use the hormone insulin is affected [10], leading to elevated levels of insulin in blood (hyperinsulinemia) and consequently, when the pancreas does not have the ability to secrete more insulin to maintain normoglycemia, results in an impaired peripheral glucose utilization (hyperglycaemia) [11]. It is a complex metabolic abnormality, that is implicated in T2DM, obesity, hypertension, dyslipidaemia [12] and is indispensable for endothelial dysfunction in T2DM [13].

The intracellular signalling pathways critical for vascular responses, activated through insulin, are counterbalanced between phosphatidylinositol 3 kinase (PI3K)-Akt kinase, a dependent insulin signalling pathway effector, which mediate the metabolic actions of insulin and phosphorylate endothelial nitric oxide synthase (eNOS) at serine 1177 (S1177), and through mitogen activated protein kinase (MAPK), a dependent insulin signalling pathway effector, that regulates the secretion of the vasoconstrictor endothelin-1 (ET-1) [14]. In IR circumstances, disturbances in the balance of the pathway of PI3K-Akt, can cause a decrease in eNOS activity, leading to less NO generated and decreased insulin-mediated vasodilation [15]. Nevertheless ET-1 and MAPK effects persist and promote adverse vasoconstriction, a raise in reactive oxygen species (ROS) production and, prothrombotic and

proinflammatory actions, which could develop vascular disease [16], ultimately leading to endothelial dysfunction [14].

1.3 Systemic hypertension

Systemic hypertension is a main risk factor for cardiovascular diseases and is defined as high systemic arterial blood pressure [17]. In the scientific community, there is not a consensus if endothelial dysfunction is a cause or consequence of hypertension. Since endothelial dysfunction can appear at early stage of hypertension contributing this way to an augment of arterial blood pressure, or it can be a consequence and contribute to more peripheral vascular resistance and systemic cardiovascular complications, aggravating the progress of the disease [18].

It was shown in isolated arteries, from different animal models of hypertension, that the endothelium-dependent vasodilation is diminished [16]. Similarly the endothelium response to vasodilators is reduced in hypertensive humans [16]. Hypertension decreases the bioavailability of NO increasing the oxidative stress due to augmented ROS generation [19]. This reduction in NO availability has been attributed to elevated circulating levels of asymmetric dimethyl arginine (ADMA) in both hypertensive animals and humans [16].

1.4 Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a syndrome characterized by progressive obliteration of pulmonary arterioles and proliferation of pulmonary vascular endothelial and smooth muscle cells, that in the most aggravated prognostic leads to increased pulmonary vascular resistance, progressive right heart failure and death [20]. PAH has recently been associated with T2DM and was shown that T2DM patients have significantly higher prevalence of PAH [21]. Despite the fact that IR and obesity are strongly related with systemic cardiovascular diseases, a recent study suggested that these pathological features can increase the susceptibility to PAH and can be associated with the severity of the disease [20,22].

When endothelial dysfunction is present simultaneous with diabetes and PAH, there is a greater vascular production of superoxide, which in turn will react with NO, reducing NO diffusion [21,23]. In pulmonary vascular diseases associated with endothelial dysfunction, there are several evidences that point to a diminished bioavailability of L-arginine and tetrahydrobiopterin (BH4) or to higher levels of ADMA [17]. These changes lead to a decrease in the NO synthase expression, which consequently endorse the vasoconstriction and cell proliferation [24].

1.5 Endothelial Dysfunction

Endothelium is formed by a monolayer of squamous epithelium, covering the internal lumen of all blood and lymphatic vessels [25]. Three layers constitute the arterial vessels: the tunica intima, a unique layer of endothelial cells; the tunica media, that includes the vascular smooth muscle cell (VSMC); and tunica adventitia, an elastic lamina with terminal nerve fibres adjacent to connective tissue [25]. This semi-permeable membrane plays an essential role in maintaining vascular health and conserving metabolic homeostasis [26]. For a necessary normal vascular function, the endothelium, produces and releases numerous endothelium derived relaxation factors (EDRF), that regulate vascular tone, modulate immune responses, coagulation and control the vascular cell growth [27]. These EDRF include NO, endothelium derived hyperpolarizing factor, prostacyclin, angiotensin II, adhesion molecules and cytokines [25].

Any slightly disturbance in the homeostasis of the endothelium, may cause endothelial dysfunction [28], that is widely described as an imbalance between vasodilation and vasoconstricting substances, produced by endothelial cells [29]. Endothelial dysfunction is recognized as an essential intermediary for

development of cardiovascular diseases covering vascular diseases associated with diabetes and hypertension [30]. Current evidences suggest that endothelial dysfunction is linked with TD2M, through impaired endothelial-dependent vasodilation [31]. It has been recognized that systemic hypertension and PAH associated with impaired vasorelaxation [18,21], diabetes mellitus, obesity, hypercholesterolemia or sedentary lifestyle, significant enhance the risk for endothelial dysfunction, atherosclerosis and systemic cardiovascular diseases [26] (see figure 1.3). Nevertheless new reports states that changes in lifestyle plus the implementation of modulatory drug therapies, including HMG CoA reductase inhibitors, angiotensin-converting enzyme inhibitors and BH4, appears to improve, or even reverse, endothelial dysfunction [32].

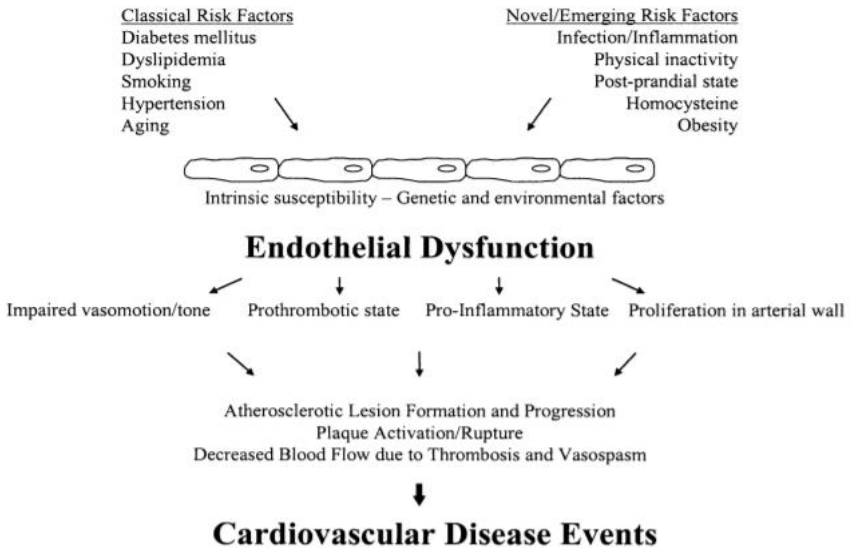

Figure 1.3-The response of the endothelium to the cumulative effects of risk factors contributing to the development and progression of cardiovascular disease events [90].

1.5.1 Vascular contractility

Vascular contractions can be initiated by the endothelium cells of the vascular smooth muscle. It was demonstrated that endothelium-derived contracting factors (EDCF) (including ET-1, thromboxane A, endoperoxides and superoxide anions [33]) are the main factors accountable for these increases in vasomotor tone. The vascular homeostasis is maintained by a subtle balance between endothelium-dependent relaxations and contractions, but when combined by a reduced production of relaxing signals and a higher secretion of EDCF this can lead to endothelium dysfunction [34].

It is well known that diabetes is associated with the accumulation of calcium in the vascular cells and with the decrease in their elasticity, which could result in the reduction of the vascular lumen and in hypertension [35]. An abnormal endothelial function or expression of potassium channels has been associated with excessive vasoconstriction [36].

ROS plays an important role in the regulation of the vascular tone [34]. This is demonstrated when the superoxide anions inactivates the EDRF-NO, which reduced the NO bioavailability and consequently increased the endothelium-dependent contractions [34].

Even though is complicate to assess all alterations that lead to endothelial dysfunction, one hallmark commonly used is to investigate whether the physiological vasodilator responses are undamaged [15]. The acetylcholine (ACh), a muscarinic cholinergic agonist, as the ability to relax VSMC indirectly by stimulating the release of EDRF [37]. The vasodilation mediated by ACh is determined by the NO produced by the endothelium [15], and an impairment in the endothelium-dependent vasorelaxation in

response to ACh, is constantly observed in human patients and experimental animal models of hypertension [18].

1.5.1.1 Prostaglandin F2α

Through the short-lived of endoperoxides, various eicosanoids are formed, such as the subclass prostanoids, consisting in prostaglandins, prostacyclins and thromboxanes [38]. Prostanoids are involved in the endothelium function, as mediators in the control of vascular tone, the remodelling of the vascular wall, in thrombosis and platelet aggregation [39], being potent vasoconstrictors the prostaglandin $F_2\alpha$ ($PGF_2\alpha$), prostaglandin B_2 and prostaglandin E_2 [40]. They have been associated in the pathogenesis of different disorders disturbing cardiac and cerebral blood flow [41]. It was demonstrated that $PGF_2\alpha$ increases pulmonary arterial pressure and pulmonary vascular resistance in lung rats [42]. $PGF_2\alpha$ receptor (FP receptor) is expressed in endothelium and in VSMC [40]. FP receptor coupled with a G_q protein, stimulate the DAG/IP_3, which is involve in calcium mobilization as well increases in calcium concentration [42], leading to the smooth muscle contraction [40,43]. The FP receptor has been associated with different conditions, such as IR, diabetes and cardiovascular complications, and it is known that the FP receptor is overexpressed in an IR state [42].

1.6 Nitric Oxide

NO is an endothelium-dependent vasodilator and has been shown to play a key role in the regulation of vascular tone, blood flow and modulation of the release of other endothelium-derived mediators [44]. NO also protects the endothelium by preventing abnormal constriction, inhibiting the aggregation of platelets, assisting the adhesion and penetration of white blood cells and releasing vasoconstrictors and mitogenic ET-1. NO is the most significant EDRF [34].

NO is produced through the enzymatic conversion of L-arginine to L-citrulline by eNOS together with the cofactor BH4 (see Figure 1.4). The enzyme activity of eNOS requires calcium, calmodulin, BH4 and nicotinamide adenine dinucleotide phosphate (NADPH) [34]. eNOS monomers constituted by the reductase domain, transfer electrons from NADPH to flavin adenine dinucleotide and flavin mononucleotide. The stimulation of the enzyme activity, occurs by shear stress and by the activation of other receptors-that mediate the action of different stimulus like insulin, oestrogens, vascular endothelial growth factor and ACh [16].

Tree isoforms of NO synthase have been identified: two constitutive isoforms, the neuronal nitric oxide synthase (nNOS), that produces NO that act as a neuronal messenger regulating synaptic neuronal transmitter release, and the eNOS, that is mainly expressed in endothelial cells; and one inducible nitric oxide synthase (iNOS) which is calcium-independent and is only expressed in cells that were previously exposed to injuries, activating macrophages or inflammatory mediators [44]. eNOS is the main enzyme isoform responsible of circulating NO in physiological conditions [25].

NO diffuses into the VSMC, by activating soluble guanylyl cyclase, then converting guanosine-5'-triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), in the VSMC [31]. This increase in cGMP, activates cGMP-dependent protein kinase, which endorses an increased extrusion of Ca^{2+} ions from VSMC, inhibiting the contractile machinery and initiating the vascular relaxation [25] (see Figure 1.4). Therefore, problems in NO synthesis, and therefore its availability to allow vascular relaxation, may be a significant cause in the pathogenesis of hypertension [45].

In fact, a decrease in the bioavailability of NO is an important factor to endothelial dysfunction and atherosclerosis, and it is well demonstrated that could be caused through different mechanisms, such as the absence of enzymatic cofactors for eNOS, the inactivation of NO by the reaction of superoxide (O_2^-) culminating in peroxynitrite anion ($ONOO^-$) formation, through the effect of superoxide NADPH oxidase and by competitive binding to eNOS [46].

NO is available when exists a balance between NO production through eNOS and ROS inactivation [47]. The hyperglycaemia in DM has been proved to develop endothelial dysfunction by enhancing oxidative stress, which uncouples the mitochondrial oxidative phosphorylation and eNOS activity, resulting in a decrease in NO availability and in the production of more ROS [47]. It has also been shown that the diminished endothelium-dependent vasodilation in diabetic subjects, results from the inactivation of NO due to the increased of oxidative stress, instead of the decreased of NO production from vascular endothelium, which this anomalous NO metabolism is associated to severe diabetic microvascular complications [25]. It is well known that the synthesis of BH_4 is rate limited by GTP cyclohydrolase, so when this pathway is absent, electron transport through eNOS can become uncoupled, resulting in O_2^- generation [15]. In addition, ROS can cause *S*-glutathionylation of eNOS leading to the inactivation of the enzyme [16].

Although the main precursor of NO is L-arginine, NO cannot be limited by the circulation of L-arginine since the regulation of NO production is multifactorial. However is limited by a metabolite of L-arginine, ADMA (an endogenous competitive inhibitor), that decreases NO production by competing with L-arginine for the binding to eNOS, contributing to NO deficiency [48]. In isolated arteries from animal models of systemic hypertension and PHA, the endothelium-dependent relaxations are decreased [34]. This has been attributed to a reduced NO bioavailability due to higher levels of ADMA [34,17]. L-arginase can also be metabolized by arginase, and by competing with the same substrate arginase is an important regulator of NO bioavailability, and can be involved, in this way, in endothelial dysfunction [25].

Endothelial function as shown to be improved through the supplementation with BH4 or its precursor sepiapterin (restores the endothelium-dependent relaxation [34]) or by the reduction of oxidative stress by targeting uncoupled NOS in patients with hypertension, diabetes, hypercholesterolemia and coronary artery disease [46]. In some studies, where diabetic vessels have been tested *in vivo*, enhancements in NO-mediated vasodilation in response to anti-oxidants like superoxide dismutase have been demonstrated [31]. Moreover it was also established that insulin can enhance the expression of eNOS in native endothelial cell *in vitro* and assists NO-dependent vasodilation *in vivo* and *in vitro* [34].

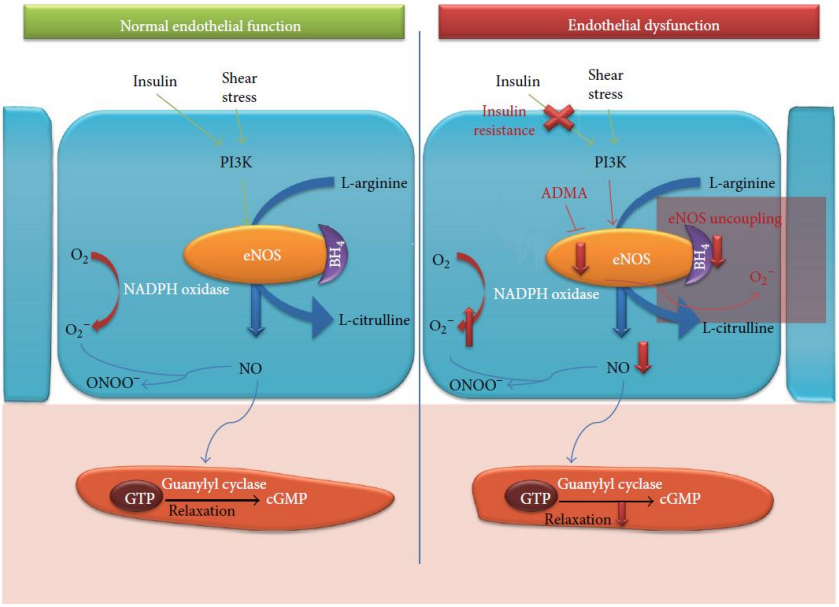

Figure 1.4- Normal endothelial function vs endothelial dysfunction. In a normal endothelial vessel, the response to insulin signaling or shear stress, through PI3K pathway is activated to phosphorylate eNOS. eNOS associated with BH_4 converts L-arginine to L-citrulline producing NO. NO interacts with guanylyl cyclase, which increases the production of cGMP through GTP, inducing VSMC relaxation. In an overview of endothelial dysfunction, insulin resistance impaired insulin-mediated NO

production which in turn leads to a decrease in vasodilation. Superoxide reacts with NO to form peroxynitrite reducing NO bioavailability that has oxidant properties. ADMA acts as an endogenous competitive inhibitor. Furthermore in a situation where the BH_4 is diminished, eNOS became uncoupled which increased ROS generation that ultimately contributes to reduce bioavailability of NO and relaxation (adapted from [19]).

1.6.1 eNOS

eNOS is localized in the luminal cell membrane of vascular endothelial cells [17] and has different physiological roles in vascular homeostasis, such as the regulation of vascular tone (key mediator of endothelium-dependent vasodilation), prevention of endothelial cell apoptosis, inhibition of platelet aggregation and, downregulation of chemoattractant proteins and inhibition of growth factors [16].

eNOS activation is endorsed by the interaction with heat shock protein 90, this protein can displace the inhibitory protein caveolin-1 from eNOS [15,16]. eNOS can be phosphorylated at several serine and threonine residues, however the phosphorylation of S117 appears to be an important regulator of its enzymatic activity augmenting the electron flux and impairing calmodulin dissociation, resulting in an overactivation of eNOS and subsequently in an increase in the NO production [15].

Some studies have shown that eNOS-deficient animals develop insulin resistance and metabolic abnormalities, similar to what is observed in T2DM [16]. Variants of eNOS polymorphisms may participate in the pathogenic pathway developing to diabetic vascular complications [25], such as hypertension, increased diet-induced atherosclerosis and increased VSMC proliferation in response to vessel damage [15]. Although the possible mechanisms of decreased NO bioavailability comprises an impairment in eNOS mRNA or protein expression levels, some studies in animal models and humans proposed that the decrement in the amount of total eNOS is not essentially associated with diabetes and atherosclerosis [15].

1.6.2 iNOS

Many cell types, including macrophages, chondrocytes, Kupffer cells, hepatocytes, neutrophils and pulmonary epithelium [49], expressed iNOS to act as a host defence against microbial and viral pathogens [50]. In response to a wide display stimuli, like endogenous pro-inflammatory mediators and endotoxins, iNOS produces NO [49]. In particular, the iNOS expression in the macrophages, is activated by pathologies of inflammatory diseases, including diabetes, atherosclerosis and multiple sclerosis [50]. The activity of iNOS is essentially regulated at a transcriptional level and when the enzyme is expressed it produces enormous amounts of NO [23].

iNOS has emerged as a key protein in IR and obesity, which has been associated with cardiac contractile dysfunction and vascular complications resulting from IR [23]. Several evidences suggests that interleukin-1β, interferon-γ and tumour necrosis factor-α (TNF-α) induces the overexpression of iNOS in β-cells, resulting in an overproduction of NO causing cytotoxicity of β-cells, which can indicate an important role of NO in diabetes [51].

1.7 Carotid body

The carotid bodies (CB) are chemoreceptor organs, classically defined as O_2 sensors, and are located bilaterally at the bifurcation of the common carotid arteries, being a conglomeration of type I and type II cells. The CBs are highly vascularized with a rich blood flow and with an efferent and afferent innervation [52]. This characteristics allow them to provide a matrix for blood vessels and nerves, with a protective surrounding capsule of connective tissue [52]. The CBs information is transferred to its sensitive nerve, the carotid sinus nerve (CSN), which its sensory nerve endings penetrate the CB [53]. CSN activity is integrated in the brainstem, aiming to normalise blood gases via hyperventilation and regulate blood pressure and cardiac performance through an increase in the activation of the sympathetic nervous system (SNS) [54].

As state in the previous paragraph, the CBs are the main peripheral oxygen sensors [55], being also able to transduce other physical-chemical stimulus including, pH levels, temperature, osmolarity [52]. Additionally, recently the CBs has been proposed to be a glucose sensor, however this is not consensual [56]. The type I cells, also known as glomus, are the key pieces in the peripheral oxygen sensing [57]. On the other hand, type II cells, or sustentacular cells, have been suggested to be an adult neural stem cells sustaining neurogenesis, *in vivo*, in response to physiological stimuli [58] (see Figure 1.5). The NO has been proposed to be an inhibitor modulator of the CB chemosensory activity, by controlling the vascular tone and by modifying the excitability of glomus cells and petrosal neurons [59].

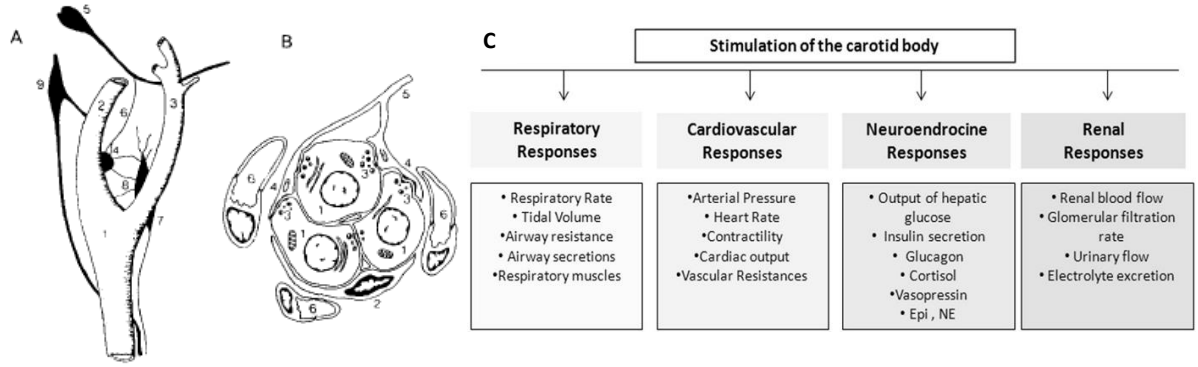

Figure 1.5- Carotid artery bifurcation (A), structured of a cellular cluster of the carotid body (B) and stimulation of carotid body produces several responses (C). A. The common carotid artery (1) splits in the internal (2) and external (3) carotid arteries. The petrosal ganglion (5), via the carotid sinus nerve (6), provides the sensory innervation to the carotid body (4). Via the ganglioglomerular nerves (8), the superior cervical ganglion (7) can innervate the carotid sinus region. The nodose ganglion (9) is located externally to the carotid artery bifurcation. **B.** The cluster of cells is formed by chemoreceptor cells (1) and surrounded by sustencular cell (2). Chemoreceptor cells are in contact with sensory nerve endings (4) of the carotid sinus nerve (5). The clusters of cells are involved by a net of capillaries [53]. **C.** The stimulation of the carotid body produces respiratory, cardiovascular, neuroendocrine and renal responses [58].

Recently, it was been demonstrated that insulin can trigger the peripheral chemoreceptors located at CB, suggesting that hyperinsulinemia may cause the CB overactivity seen in metabolic disorders, as metabolic syndrome and T2DM [60]. In fact, Ribeiro *et al.,* (2013) demonstrated that in models of metabolic syndrome and prediabetes occurs an overactivation of the CB, that results in the development of IR and hypertension, they also displayed that the CSN chronic denervation prevents the development of IR and hypertension in hypercaloric animal models [60]. Additionally, Sacramento *et al.*, (2017) showed that chronic bilateral CSN resection, in pre-existing metabolic diseases, restores insulin sensitivity and glucose tolerance as well as fasting plasma glucose, insulin levels and blood pressure, that were fully restored [61].

The involvement of the CB in the control of glucose homeostasis was supported by clinical data in T2DM patients, in where hyperbaric oxygen therapy, that functionally blocks of the CB, improve post-prandial glucose tolerance and reduced fasting glycaemia [61].

2. Aims

Knowing that endothelial dysfunction can result from and/or contribute to several disease processes, as occurs in hypertension and T2DM and that CB overactivation is associated with the development of such diseases, the general aim of the present thesis was to evaluate the impact of CSN denervation on endothelial function in the pulmonary artery and in the systemic vasculature.

The specific aims of this work were to investigate, in a model of IR, glucose intolerance and hypertension, the effect of CSN resection in the pulmonary artery and aorta on:

1. the contractile responses to an unspecific stimulus (high K^+) and to $PGF_2\alpha$
2. the vasodilation responses to ACh
3. the NO levels in plasma
4. the expression of eNOS, iNOS, and $PGF_2\alpha$ R

3. Methods

3.1 Animals and experiments

Experiments were performed in 8 weeks old male Wistar rats (200-300g), obtained from the vivarium of NOVA Medical School, Faculdade de Ciências Médicas. Animals were housed in a controlled environment (21±1°C; 55±10% humidity) with a 12-hour light/dark cycle and free access to food and water. An early-phase type 2 diabetes model (combined IR, hyperinsulinemia and increased total fat mass [62]) with hypertension was obtained by submitting animals to a high-fat and high-sucrose (HFHSu) diet was used. The HFHSu model was obtained by a combination of a lipid-rich diet with 60% of energy from fat (34% fat plus 23% protein plus 5% fibres plus 5, 50% ash; Mucedola, Italy) and 35% sucrose in drinking water during 25 weeks. This animal model was compared with an age-matched control group (CTL), fed a standard diet (7.4% fat plus 75% carbohydrate [4% sugar] plus 17% protein, SDS diets RM1; Probiológica, Lisbon, Portugal). Body weight was weekly recorded meanwhile caloric and liquid intake were monitored daily in all groups of animals.

After 14 weeks of diet, IR was confirmed by an insulin tolerance test (ITT) and glucose tolerance through oral glucose tolerance test (OGTT). Subsequently, the HFHSu and CTL groups were randomly divided and half of the group of animals was submitted to a bilateral CSN resection under a mixture of ketamine (30mg/kg)/medetomidine (4mg/kg) anaesthesia and carprofen (5mg/kg), a non-steroidal anti-inflammatory drug. Atipamezole (2mg/kg) was used to reverse the effects of the sedative. One day post-surgery was administered buprenorphine (10µg/kg), an opioid agonist, and during the three days' post-surgery carprofen (5mg/kg). After the surgery, the animals were kept under the respective HFHSu and standard diet. The experimental groups were compared with sham groups that were submitted to the same surgical procedure but without resection of the CSN.

Two weeks and eleven weeks post-CSN resection, insulin sensitivity and glucose tolerance have been evaluated and afterwards at the eleven weeks' post-surgery, the rats were submitted to a terminal experiment in which the animals were anesthetized with pentobarbital (60mg/kg, i.p.). After anaesthesia, the femoral artery was catheterized and mean blood pressure (MBP) was recorded using a pressure transducer (-50, +300 mmHg, emka, Paris, France) and a pressure amplifier (emka, Paris, France). The MBP was analysed though the software IOX 2.9.5.73 (emka, Paris, France). Blood was collected though a cardiac puncture to EDTA-precoated tubes and then processed into plasma for measuring NO levels by chemiluminescence. The aorta and the pulmonary arteries were excised and dissected for vascular function studies (Myography) or stored for later analyses (Western Blot and NO quantification) at -80ºC. The contractile response of the arteries was evaluated in response to increasing doses of $PGF_2\alpha$ by myography. When a later steady state tonic response was attained with $PGF_2\alpha$, cumulative increasing concentrations of ACh were added to induce an endothelium-dependent relaxation, obtaining a concentration–response curve (CRC) and therefore evaluating endothelial dysfunction. NO levels in the aorta and pulmonary artery were quantified after their homogenization by chemiluminescence, while the expression of eNOS, iNOS and $PGF_2\alpha$ receptor (FP receptor) were obtained by Western Blot.

All experiments and animal care were performed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU), and all the experimental protocols were approved by the ethics committee of the NOVA Medical School/Faculdade de Ciências Médicas.

3.1 Insulin Tolerance Test

The ITT assess insulin sensitivity in conscious animals and consist in the administration of a bolus of insulin in the tail vein (0.1 U/kg body weight), after an overnight fasting [12]. The plasma glucose decrement was measured over 15 min at 1 min intervals. Blood was collected via tail tipping and

glucose levels were measured with a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal) [62]. The constant rate for plasma glucose decline (K_{ITT}) was calculated through the product of 0.693 by the plasma glucose half-time (t1/2). Glucose half-time was calculated from the gradient of the least-square analysis of plasma glucose concentrations during the linear decay phase [60].

3.2 Oral Glucose Tolerance Test

OGTT provides an evaluation of the insulin released and the sensitivity of the peripheral tissue toward the insulin action [12]. After an overnight fast, all animals ingested a dose of glucose (2g/Kg) in sterile saline (0.9% NaCl) by oral gavage and subsequently plasma glucose were measure before (0 min) and 15, 30, 60, 120 and 180 minutes after the administration of glucose [63]. The glucose tolerance was measured by the area under the curve (AUC) of glucose excursion curves [12]. The procedures for collecting blood and measuring the glucose levels were identical of the ITT test.

3.3 Evaluation of endothelial function

The physiological function of the arteries was studied using a small vessel wire myograph (DMT, Denmark), technique that allows *ex vivo* monitoring of isometric tension in response to different pathophysiological stimuli [64]. The bath chambers for isolated organs were gassed with normoxia: (21% O_2 + 5% CO_2) and the temperature maintained at 37ºC. The pulmonary artery and aorta were dissected in cold Krebs-Henseleit buffer (PSS) (in mM: 118 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgSO_4$ (anhydrous), 24 $NaHCO_3$, 0.43 NaH_2PO_4 and 5.56 Glucose). Rings of aorta and pulmonary artery (inner diameter: 0.5-1.0 mm) were dissected under a dissection microscope and were withdrawn of all adventitia and parenchyma. The arteries were connected to isometric force transducers to measure changes in isometric tension and then stretched to give a basal tension of 5-6mN. After stabilization, to check the viability of the vessels, three responses of physiological salt solution (KPSS) (in mM: 38 NaCl, 80 KCl, 1.8 $CaCl_2$, 1 $MgSO_4$ (anhydrous), 24 $NaHCO_3$, 0.5 NaH_2PO_4 and 5.56 Glucose) were performed, washing twice with Krebs-Henseleit buffer between them. To constrict the vessels increasing doses of $PGF_2\alpha$ (0.03µM-10/30µM) were added to the chamber solution. Twenty minutes after washing and on a stable $PGF_2\alpha$ contraction, a cumulative CRC to ACh (0.03µM-30µM), an endothelium-dependent vasorelaxant, was performed to determine endothelial integrity, washing twice afterwards.

3.4 Nitric Oxide Quantification

To quantify nitric oxide in aorta and pulmonary artery, the arteries were previously dissected and homogenized in a glass tissue homogenizer with buffer (25mM Tris HCL, 1mM EDTA, 1mM EGTA). Afterwards, the homogenates were centrifuged at 1300g (4ºC) for 20 minutes and the supernatant collected. To quantify NO in plasma, blood was collected though a cardiac puncture to EDTA-precoated tubes and centrifuged at 3000g (4ºC) during 10 min. Samples deproteinization was accomplished by diluting the plasma with ethanol absolute at 4ºC (1:3), leaving it in ice for 30 minutes, centrifuged at 1200g (4ºC) for 15 minutes and lastly collecting the supernatant. NO levels were quantified using the Sievers Nitric Oxide Analyser (NOA 280i; Sievers Research Inc., Boulder Colorado, USA), which is a reliable detection system for assess NO levels and its metabolites in the liquid system (e.g., plasma), that allows the interaction between NO and ozone to elicit chemiluminescence [65]. The preparation the NOA consists in turning the bath at 90ºC, adding NaOH (1 M) until the filter is covered, then adding a vanadium solution (50 mM VaCl3 in 1 M HCl) through the injection port and waiting until the baseline is stable (10-15 mV). The vanadium starts to bubble when the cell press is adequate (5 - 7.4 Torr). All samples were injected with a Hamilton syringe (5µl). NO concentrations were calculated by comparison with an interpolation of a calibration curve made from increasing concentrations of sodium nitrate (1µM, 2µM, 5µM, 10µM, 20µM, 50µM).

3.5 Western Blot analyses of eNOS, iNOS, $PGF_2\alpha$ R in aorta and pulmonary artery

For western blot analysis, pulmonary artery (from the large lobe of the left lung) and aorta were dissected and homogenized in liquid nitrogen and then placed in Zurich medium (10mM Tris–HCl; 1mM EDTA; 150 mM NaCl; 1% Triton X-100; 1% sodium cholate; 0.1% sodium dodecyl sulfate) containing aprotonin (1mg/ml), leupeptin (1mg/ml), pepstatin (1mg/ml), tripsin (1mg/ml), Na3VO4 (100mM) and PMSF (100mM). Protein concentrations of the homogenates were measured with a Micro-BCA Protein Assay Kit (Pierce, Madrid, Spain). Calculations were performed to use equal volumes in each well (protein + Zurich medium + sample buffer (50 % µl of sample buffer + 50 % µl of mercaptoethanol)). Denaturation of samples was done at 99ºC for 5 minutes followed by a centrifugation of 5 minutes at 8000 rpm (4ºC). Proteins (20µg) and molecular weight marker (Precision Plus Protein, BioRad, USA) were separated by electrophoresis in 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and afterwards a transferred to a nitrocellulose membrane (0.2 µM, BioRad, Germany). A Red Ponceau (0.1%) staining was performed for 5 min with agitation to verify the quality of the transfer. The membranes were blocked in I-Block solution (0.5%) (Applied Biosystems, Foster City, USA) for 1h with agitation at room temperature. A three-step Western blot protocol was used to enhance detection sensitivity [66]. After blocking, membranes were incubated overnight at 4°C with primary polyclonal rabbit anti-eNOS antibody (bands in the 140 kDa region, 1:500; Santa Cruz Biotechnology, Madrid, Spain), primary polyclonal rabbit anti-iNOS antibody (bands in the 130 kDa region, 1:200; Santa Cruz Biotechnology, Madrid, Spain) or, primary polyclonal rabbit anti-FP receptor antibody (bands in the 45 kDa region, 1:200; Santa Cruz Biotechnology, Madrid, Spain). Afterwards the membranes were incubated with biotin-conjugated goat anti-rabbit IgG (1:5000) in Tris-buffered saline with Tween 20 (TBST) (0.1%) for 2h and horseradish peroxidase-conjugated streptavidin (1:10000) in TBST (0.1%) for 30min at room temperature. Between incubations, the membranes were washed with TBST (0.02%) 4 times of 10 min. Chemiluminescence signals were developed with enhanced chemiluminescence reagent (Clarity Western ECL, Bio-Rad, United States), the signal detected in a Chemidoc Molecular Imager (Chemidoc; BioRad, Madrid, Spain) and quantified using the Quantity-One software (BioRad). The membranes were reprobed with polyclonal goat anti-calnexin (bands in the 90 kDa region, 1:1000; SicGen, Portugal) to compare and normalize the expression of proteins with the amount of protein loaded.

3.6 Statistical analysis

Data was evaluated using GraphPad Prism Software, version 6 (GraphPad Software Inc., San Diego, CA, USA) and was expressed as mean ± SEM. The significance of the differences between the means was calculated by one-way and two-way analysis of variance (ANOVA) with Bonferroni multiple comparison tests. Differences were considered significant at $p \leq 0.05$.

4. Results

4.1 Effect of HFHSu diet and CSN resection in insulin sensitivity

Figure 4.1 represents the effect of HFHSu diet and CSN resection on insulin sensitivity, determined by the ITT, before diet and at fourteen, sixteen and twenty-five weeks of diet. As it can be seen and expected (Figure 4.1), HFHSu diet decreased insulin sensitivity (K_{ITT} HFHSu baseline = 4.36±0.25; K_{ITT} HFHSu 14 weeks diet = 1.67±0.5; K_{ITT} HFHSu 16 weeks diet = 1.93±0.59; K_{ITT} HFHSu 25 weeks of diet = 1.35±0.78 % glucose/min; K_{ITT} CTL baseline = 4.36±0.56; K_{ITT} CTL 14 weeks diet = 4.092±0.41; K_{ITT} CTL 16 weeks diet = 4.26±0.29; K_{ITT} CTL 25 weeks of diet = 4.21±0.52% glucose/min). CSN resection did not affect significantly the insulin sensitivity in control animals (K_{ITT} CTL 16 weeks' diet with CSN resection= 3.46±0.87; K_{ITT} CTL 25 weeks of diet with CSN resection = 4.34±0.53 % glucose/min). After CSN resection in HFHSu animals, insulin sensitivity increased by 81% 2 weeks post-surgery (K_{ITT} = 3.87±0.61% glucose/min) and 111% 11-weeks post-surgery (K_{ITT} = 4.51±0.36% glucose/min) in comparison with the value of insulin sensitivity before surgery (K_{ITT} = 2.13±1.25% glucose/min).

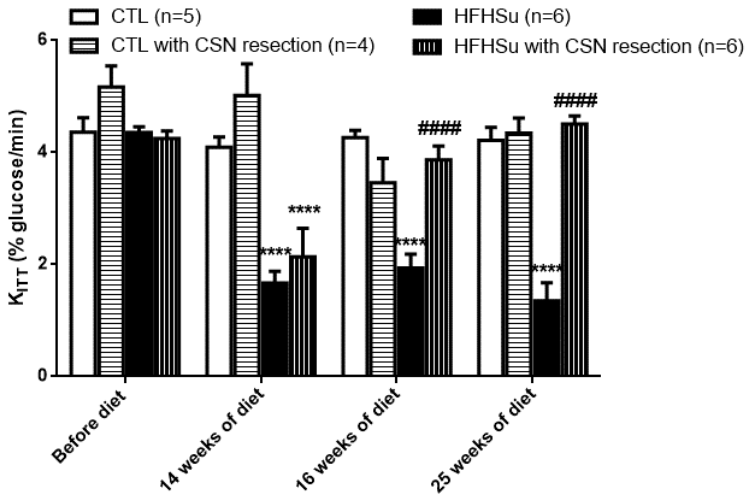

Figure 4.1- Effect of HFHSu diet and of CSN resection in insulin sensitivity assessed by the ITT and expressed by the K_{ITT}, the constant obtained from the ITT. Bars represents mean ± SEM. Two-away ANOVA with Bonferroni multiple comparisons tests; **** p<0.0001 vs CTL; #### p<0.0001 comparing values with HF HSu.

4.2 Effect of HFHSu diet and CSN resection on glucose tolerance

Figure 4.2 illustrates the effect of HFHSu diet and of CSN resection on glucose tolerance, expressed as the AUC of the glucose excursion curves obtained from the OGTT before diet and at fourteen, sixteen and twenty-five weeks of diet. The standard diet did not alter glucose tolerance throughout the weeks of diet, however as expected HFHSu diet increased significantly the AUC of the glucose excursion curves, suggesting the development of glucose intolerance (AUC HFHSu baseline = 21446±475; AUC HFHSu14 weeks diet = 24900±602; AUC HFHSu 16 weeks diet = 23425±518; AUC HFHSu 25 weeks of diet = 24334±722 mg/dl*min; AUC CTL baseline = 21459±735; AUC CTL 14 weeks diet = 20343±566; AUC CTL 16 weeks diet = 19951±682; AUC CTL 25 weeks of diet = 21897±715 % mg/dl*min). CSN resection at 14 weeks of diet did not alter glucose tolerance in CTL animals (AUC CTL 16 weeks diet with CSN resection = 19867±469; AUC CTL 25 weeks of diet with CSN resection = 19297±1283 mg/dl*min). At the fourteen weeks of diet, the animal's HFHSu (24900±602 mg/dl*min) and HF HSu with CSN resection (23991±445 mg/dl*min) augmented the glucose intolerance by 22% and 18%, respectively, in relation to CTL AUC= 20343±566 mg/dl*min. After

twenty-five weeks of diet, the values of HFHSu with CSN resection (2214±928 mg/dl*min), were brought back to CTL levels AUC=21897±715 mg/dl*min.

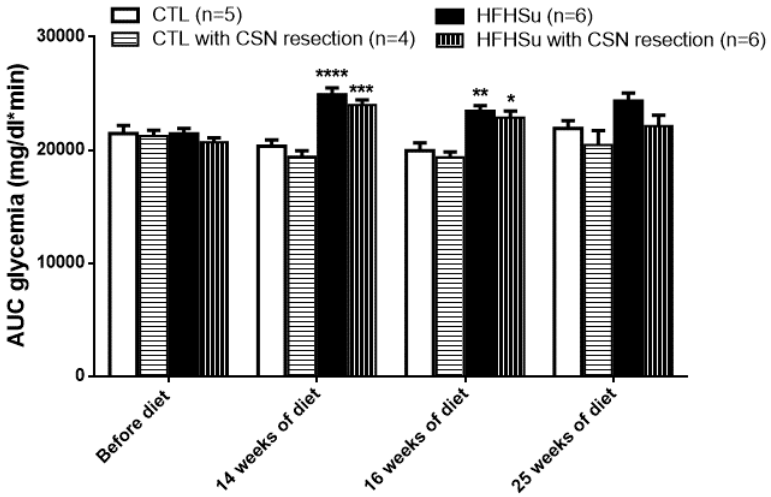

Figure 4.2- Effect of HFHSu diet and of CSN resection on glucose tolerance, expressed as the area under the curve (AUC) obtained from the glucose excursion curves determined by the OGTT. Bars represents mean ± SEM. Two-away ANOVA with Bonferroni multiple comparisons tests; * p<0.05; ** p<0.01; ***p<0.001; **** p<0.0001 vs CTL.

4.3 Effect of HFHSu diet and of CSN resection on mean blood pressure

In the figure 4.3 is demonstrated the effect of HFHSu diet and of CSN resection on MBP. The HFHSu diet increases the MBP by 52% in relation to CTL diet (CTL = 80.9±4.3mmHg; HFHSu = 123.4±1.96mmHg). CSN resection did not alter the MBP in CTL animals, but in HFHSu rats it decreases significantly by 23% (CTL CSN resection = 78.9±2.9mmHg; HFHSu CSN resection = 95.2±3mmHg).

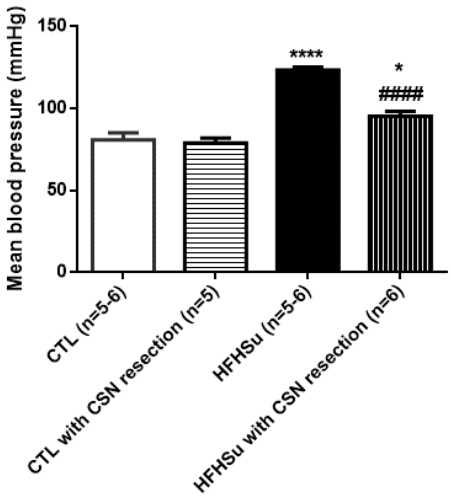

Figure 4.3-Effect of HFHSu diet and of CSN resection on mean blood pressure. Bars represents mean ± SEM. One-away ANOVA with Bonferroni multiple comparisons tests; * p<0.05; **** p<0.0001 vs CTL; #### p<0.0001 vs HFHSu.

4.4 Effect of HFHSU diet and of CSN resection on vasoconstrictor responses in pulmonary artery and aorta

The effect of HFHSU diet and of CSN resection on the contractile responses induced by KPSS (80mM) in the pulmonary artery and aorta is represented in the figure 4.4A and 4.4B respectively. HFHSu diet did not modify the contractile responses to the unspecific stimulus KPSS in both pulmonary artery and aorta (figure 4.4). CSN resection, decreased the contractile responses, in CTL

and HFHSu animals (figure 4.4), however this decrease was non-significant in the pulmonary artery (Contractile response KPSS CTL = 8.43±0.41mN; Contractile response KPSS CTL CSN resection = 6.74±0.49mN; Contractile response KPSS HFHSu = 8.95±0.54mN; Contractile response KPSS HFHSu CSN resection = 7.08±0.69mN). In the aorta, CSN resection reduced by 35% and by 26% the contractile responses to KPSS in CTL and HFHSu animals, respectively (Contractile response KPSS CTL aorta = 8.76±0.73mN; Contractile response KPSS CTL CSN resection aorta = 5.69±0.54mN; Contractile response KPSS HFHSu aorta = 9.25±0.82mN; Contractile response KPSS HFHSu CSN resection = 6.83±0.6mN).

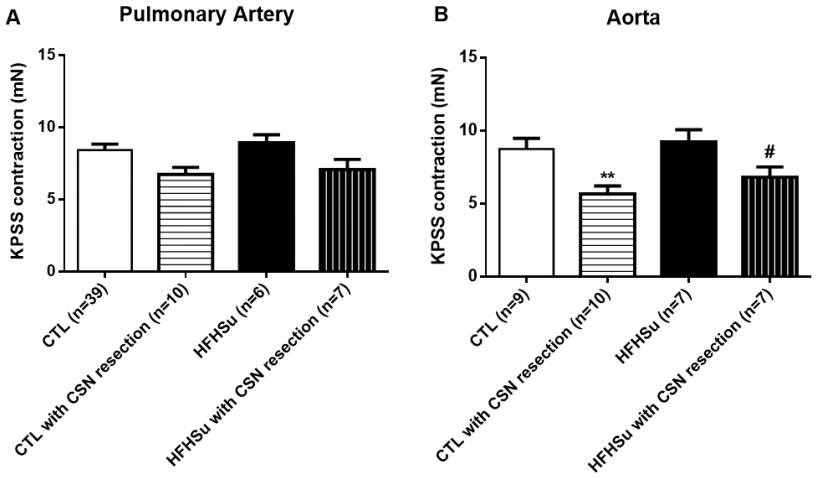

Figure 4.4- Effect of HFHSu diet and of carotid sinus nerve (CSN) resection on vasoconstrictor responses to 80mM of K^+ (KPSS) in the pulmonary artery (A) and aorta (B). Bars represents mean ± SEM. One-away ANOVA with Bonferroni multiple comparisons tests; ** p<0.01 vs CTL; # p<0.05 vs HF HSu.

4.5 Effect of HFHSu diet and of CSN resection on endothelial function of pulmonary artery and aorta

Figure 4.5 shows the contractile responses to increasing doses of $PGF_2\alpha$ (0.03μM-10/30μM) in the pulmonary artery and aorta. Note, that HFHSu diet decreased significantly the contractile responses to $PGF_2\alpha$ in the pulmonary artery (figure 4.5A). In fact, for the, highest concentration tested, 10μM, the contractile responses of the HFHSu animals were decreased by 59%, in relation to CTL values (at 10μM: CTL = 9.5±1.23%; HFHSu = 3.9±0.55%). CSN resection did not modify significantly the contractile dose-response curve for $PGF_2\alpha$ in the pulmonary artery however for the highest concentration tested (10μM) it increased by 42% (P value = 0.0552) the contractile response (HFHSu = 3.9±0.55%; HFHSu with CSN resection = 5.5±0.52%). In contrast, in the aorta, the HFHSu presented a higher contractile response to $PGF_2\alpha$ (figure 4.5B), and for the higher concentration tested, 10μM, HFHSu diet increased significantly the contractile response by 79% (contractile response to $PGF_2\alpha$ CTL = 70.6±10.4%; contractile response to $PGF_2\alpha$ HFHSu = 126.9±13.3%). CSN resection increased the contractile dose-response curve for $PGF_2\alpha$, for the highest concentration tested (10μM), by 32% and 10% in CTL and HFHSu respectively (contractile response to $PGF_2\alpha$ CTL CSN resection = 93±10%; contractile response to PGF2α HFHSu CSN resection = 139±6.1%).

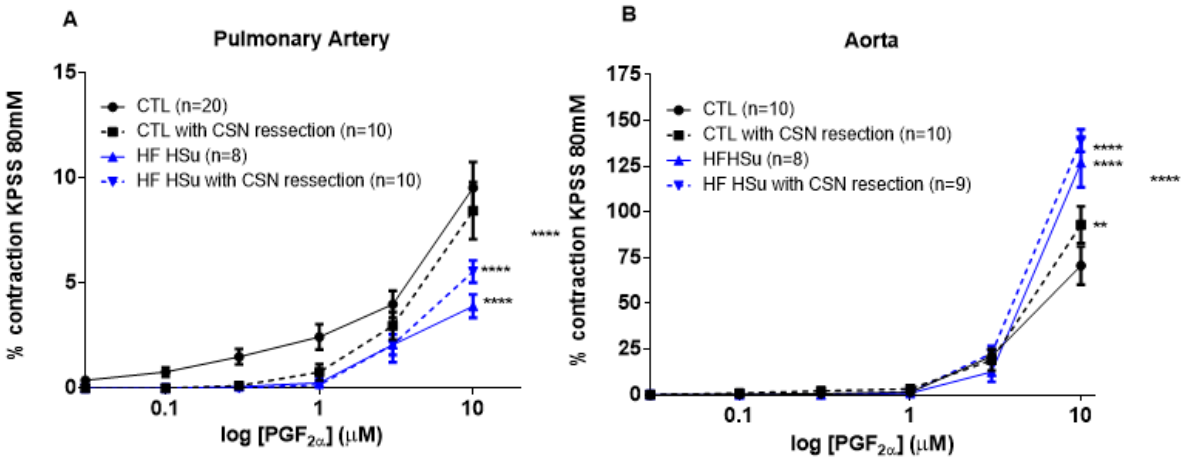

Figure 4.5- Concentration response curves for contractions to $PGF_2\alpha$ in the pulmonary artery and aorta rings. Contractile responses to $PGF_2\alpha$ in pulmonary artery (A) and aorta (B), in CTL (black line), CTL with CSN resection (black dashed line), HF HSu (blue line) and HF HSu with CSN resection (blue dashed line) groups. Two-away ANOVA with Bonferroni multiple comparisons tests; ** p<0.01; **** p<0.0001 vs CTL.

The figure 4.6 displays the evaluation of endothelial integrity, evaluated by CRC for relaxation to ACh, previously contracted with $PGF_2\alpha$ in pulmonary artery (4.6A) and in aorta (4.6B). The HFHSu diet decreased significantly the relaxation curve in both arteries. For the highest concentration tested, 30μM, the HFHSu diet decreased by 106% and 262% in the pulmonary artery and in the aorta, respectively (relaxation response to ACh at 30μM: CTL pulmonary artery = 19.5±2.65%; HFHSu pulmonary artery: 40.2±6.12%; CTL aorta = 10.1±3.19; HFHSu aorta = 36.7±4.41%). CSN resection did not modify significantly the CRC for relaxation in the CTL animals in both arteries (relaxation response to ACh at 30μM: CTL CSN resection pulmonary artery = 22.5±7%; CTL CSN resection aorta = 10.64±3.16%). However, for the highest concentration, 30μM, the CSN resection of the HFHSu animals increased by 57% and 69% in pulmonary artery and in aorta, respectively (relaxation response to ACh at 30μM: HFHSu CSN resection pulmonary artery = 17.2±5.86%; HFHSu CSN resection aorta = 11.2±1.84%).

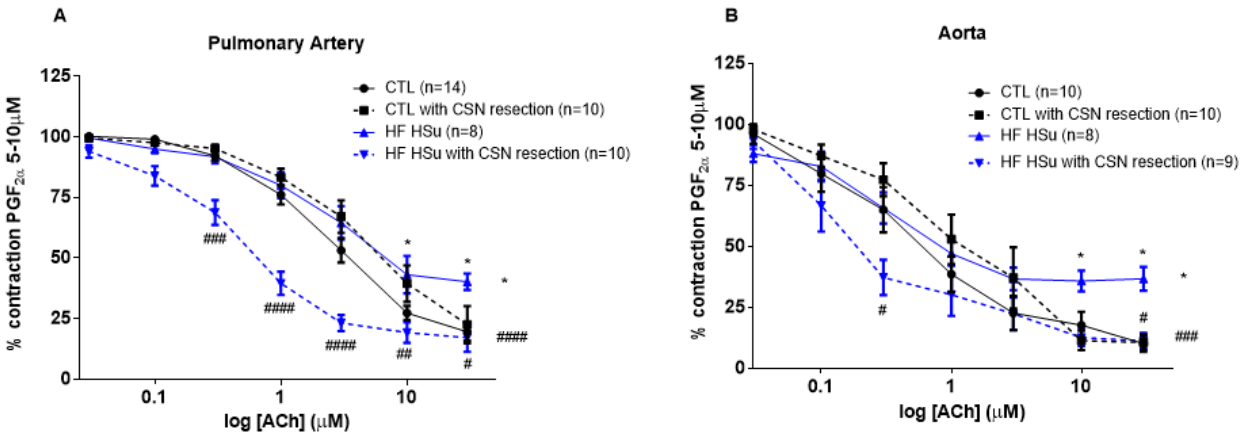

Figure 4.6- Concentration-responses curves for relaxation to ACh in pulmonary artery and aorta. Relaxation responses to ACh, in pulmonary artery (A) and aorta (B), in CTL (black line), CTL with CSN resection (black dashed line), HF HSu (blue line) and HF HSu with CSN resection (blue dashed line) groups. Two-away ANOVA with Bonferroni multiple comparisons tests; * p<0.5 vs CTL; # p<0.5, ## p<0.01, ### p<0.001, #### p<0.0001 vs HF HSu.

4.6 Effect of HFHSU diet and of CSN resection on NO levels in plasma, aorta and pulmonary artery

The effect of HFHSu diet and of CSN resection in the NO levels in plasma is presented in figure 4.7. HFHSu diet increased significantly by 74% the NO levels in plasma (NO CTL =15.34±0.4 μM; NO HFHSu = 26.67±1.64 μM). CSN resection decreased by 16% NO levels in CTL animals (NO CTL with CSN resection = 12.85±0.5 μM) and 24% in HFHSu animals (NO HFHSu with CSN resection = 20.34±0.5 μM).

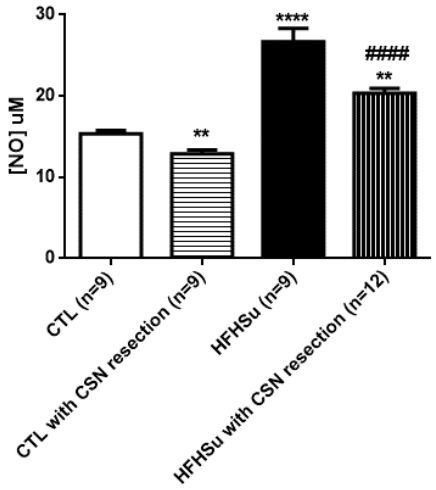

Figure 4.7- Effect of HFHSu diet and of CSN resection in NO levels in plasma. Bars represents mean ± SEM. One-away ANOVA with Bonferroni multiple comparisons tests; ** p<0.01, **** p<0.0001 vs CTL; #### p<0.0001 comparing values with HF HSu.

Figure 4.8 displays the effect of HFHSu diet and of CSN resection in NO levels in the pulmonary artery and in the aorta. HFHSu diet decreased non-significantly the NO levels in the pulmonary artery by 15% (NO HFHSu pulmonary = 249±23.7 moles/g; NO CTL pulmonary = 292±15.3 moles/g), however in the aorta HFHSu diet decreased significantly NO levels by 43% (NO HFHSu aorta = 189±35.34 moles/g; NO CTL aorta = 334±17.1 moles/g). The levels of NO in the pulmonary artery, in the HFHSu with CSN resection decreased by 25% (218±15.1 moles/g) from the control value,

however this was not statically significant when compared with the HFHSu animals (figure 4.8A). In the aorta, the NO levels of CTL with CSN resection increased by 29% (NO CTL CSN resection aorta = 430±12.67 moles/g) when compared with the control sham. However, CSN resection was unable to modify the NO levels of HFHSu rats ((NO HFHSu CSN resection aorta = 130±3.4 moles/g) (figure 4.8B).

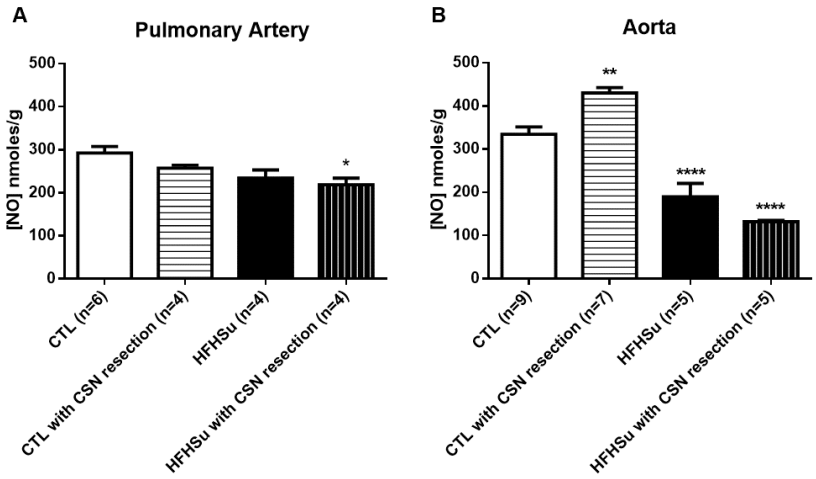

Figure 4.8- Effect of HFHSu diet and of carotid sinus nerve (CSN) resection on NO levels in pulmonary artery (A) and aorta (B). Bars represents mean ± SEM. One-away ANOVA with Bonferroni multiple comparisons tests; *p<0.05, ** p<0.01, **** p<0.0001 vs CTL.

4.7 Effect of HFHSu diet and of CSN resection on eNOS, iNOS and FP receptor expression levels in the pulmonary artery and aorta

In figure 4.9, is represented the effect of HFHSu diet and of CSN resection on eNOS expression levels in the pulmonary artery (4.9A) and in the aorta (4.9B). On the top of the graphs in the figure A and B, representative western blots comparing the expression of eNOS (140kDa) and calnexin (90kDa) in the pulmonary artery and in the aorta, are displayed. In both arteries, HFHSu diet or CSN resection did not altered significantly the expression of eNOS (Pulmonary artery: CTL = 100±3.49%; CTL with CSN resection = 105.76±7.04%; HFHSu = 94.56±9.31%; HFHSu with CSN resection = 106.57±19.93%) (Aorta: CTL = 100±3.24%; CTL with CSN resection = 96.99±7.5%; HFHSu = 89.29±5.68%; HFHSu with CSN = 107.26±2.49%).

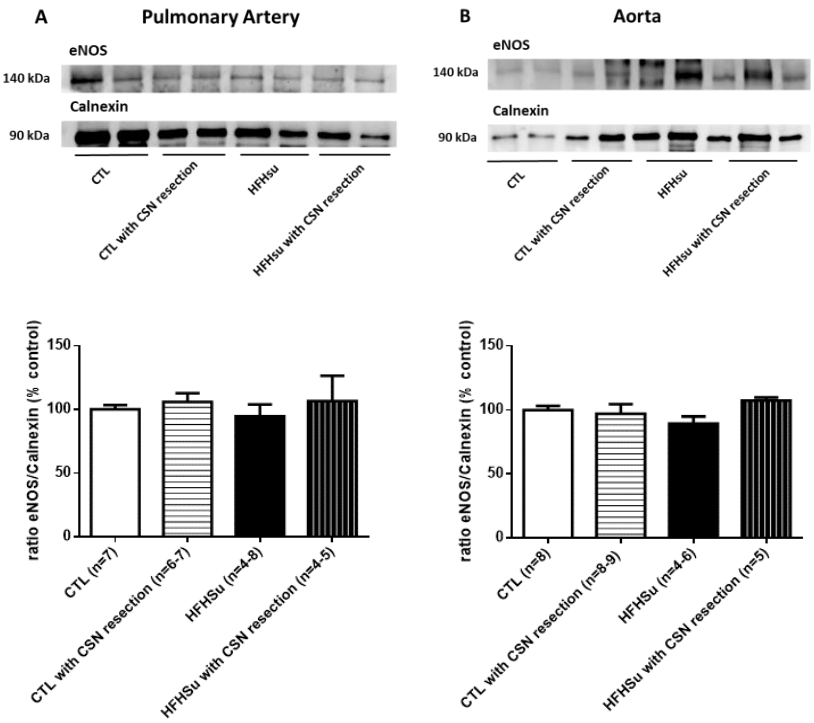

Figure 4.9 - Effect of HFHSu diet and of carotid sinus nerve (CSN) resection on eNOS expression levels in the pulmonary artery (A) and aorta (B). On the top of the panels A and B representative images of western blot for eNOS (140kDa) and calnexin (90kDa) expression for pulmonary artery and aorta. Graphs A and B represent the mean values for the expression of eNOS in the pulmonary artery and aorta, respectively, expressed in relation to calnexin, the loading protein. Bars represents mean ± SEM. One-away ANOVA with Bonferroni multiple comparisons tests.

Figure 4.10 shows the effect of HFHSu diet and of CSN resection on the expression of iNOS, in the pulmonary artery and in the aorta. On the top of panels A and B representative western blots for iNOS (130kDa) and calnexin (90kDa) in pulmonary artery and in aorta, respectively are presented. When plotted the results, it can be seen, that HFHSu diet increased significantly by 87% and by 44% the expression of iNOS in the pulmonary artery and in aorta, respectively (CTL pulmonary artery = 100±5.24%; HFHSu pulmonary artery = 187.41±21.29%; CTL aorta = 100±3.84%; HFHSu aorta = 144.49±14.45%). Surprisingly, CSN resection increased iNOS expression in the animals in the pulmonary artery by 55% (CTL = 100±5.24%; CTL CSN resection = 155.68±9%). However, in HFHSu animals CSN resection decreased significantly the iNOS expression by 36% (HFHSu pulmonary artery = 187.41±21.29%; HFHSu CSN resection pulmonary artery = 119.47±18%) (figure 4.10A). In the aorta, CSN resection did not modify iNOS expression in CTL animal's (CTL aorta = 100±3.84%; CTL CSN resection aorta = 103.36±4.93%), but decreased significantly by 53% the iNOS expression in HFHSu animals (HFHSu aorta = 144.49±14.45%; HFHSu with CSN resection aorta = 68±9.89%) to levels even below the control levels (figure 4.10B).

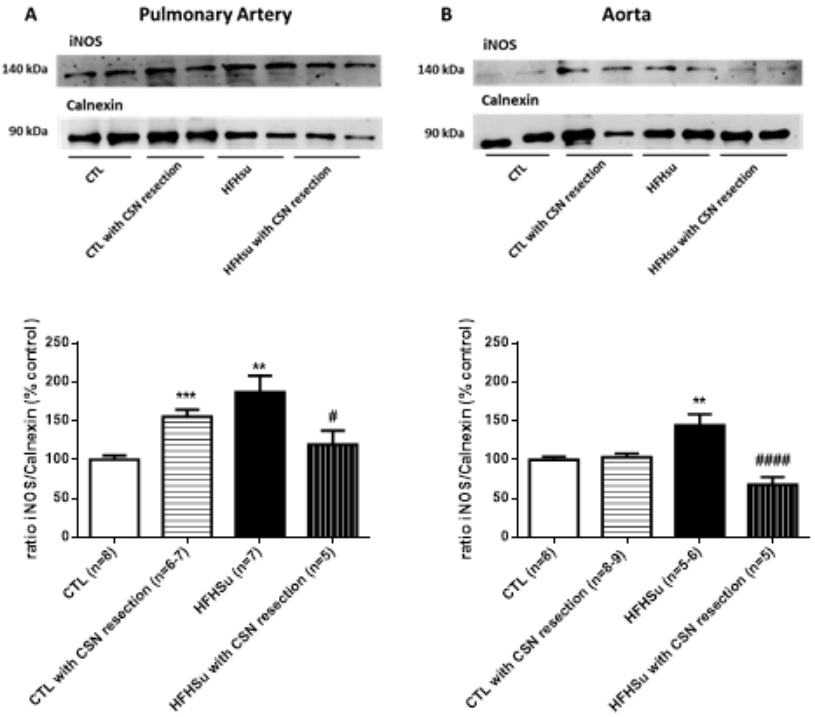

Figure 4.10 - Effect of HFHSu diet and of carotid sinus nerve (CSN) resection on iNOS expression levels in the pulmonary artery (A) and aorta (B). The top of panels A and B display representative images of western blot for the expression of iNOS (140kDa) and calnexin (90kDa), the loading protein, in the pulmonary artery and aorta, respectively. Graphs on A and B represent the mean values for the expression of iNOS in the pulmonary artery and aorta, respectively, expressed in relation to calnexin. Bars represents mean ± SEM. One-away ANOVA with Bonferroni multiple comparisons tests; ** p<0.01, *** p<0.001 vs CTL; # p<0.05, #### p<0.0001 comparing values with HF HSu.

Figure 4.11 shows the effect of HFHSu diet and of CSN resection on the expression of FP receptor levels, in the pulmonary artery and in the aorta. On the top of panels A and B, representative western blot comparing the FP receptor (45kDa) with calnexin (90kDa) in all groups of animals in the pulmonary artery and aorta, respectively, are presented. HFHSu diet increased significantly by 126% FP receptor expression in the pulmonary artery (CTL pulmonary artery = 100±6.94%, HFHSu pulmonary artery = 226.23±16.08%), without altering significantly the FP receptor expression in the aorta (CTL aorta = 100±6.62%, HFHSu aorta = 112.74±16.07%). CSN resection increased significantly by 48% the FP receptor expression at the pulmonary artery in control animals (CTL

pulmonary artery = 100±6.94%; CTL with CSN resection = 148.67±14.64%), however in the HFHSu animals it decreased by 45% (HFHSu pulmonary artery = 226.23±16.08%; HFHSu CSN resection pulmonary artery = 123.48±24.5%) (figure 4.11A). In the aorta, CSN decreased significantly by 34% the FP receptor expression in control animals (CTL aorta = 100±6.62%, CTL with CSN resection aorta = 65.78±6.68%), but did not change significantly FP receptor expression in HFHSu animals (HFHSu aorta = 112.74±16.07%; HFHSu CSN resection aorta = 126.32±8.25%) (figure 4.11B).

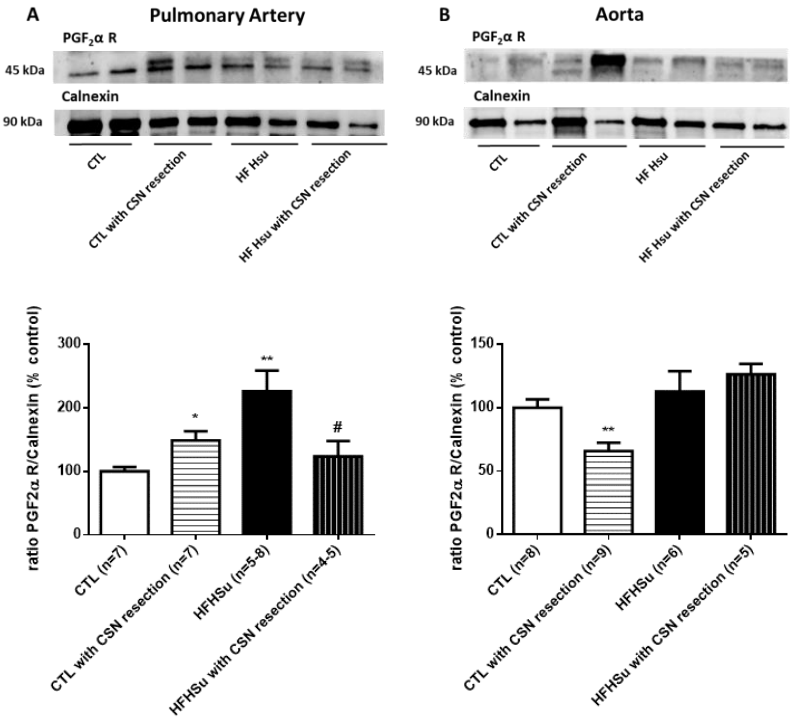

Figure 4.11 - Effect of HFHSu diet and of carotid sinus nerve (CSN) resection on FP receptor expression levels in the pulmonary artery (A) and aorta (B). The top of panels A and B display representative images of western blot for the expression of FP receptor (140kDa) and calnexin (90kDa), the loading protein, in the pulmonary artery and aorta, respectively. Graphs on A and B represent the mean values for the expression of FP receptor in the pulmonary artery and aorta, respectively, expressed in relation to calnexin. Bars represents mean ± SEM. One-away ANOVA with Bonferroni multiple comparisons tests; *p<0.05, ** p<0.01 vs CTL; #<p0.5 vs HFHSu.

5. Discussion

The major finding of the present study is that for the first time we demonstrate that CSN resection restores endothelial function in the pulmonary artery and in the aorta. Furthermore, we also show that CSN resection decreases the NO levels in plasma. Additionally, CSN resection was able to restore normal levels of expression of iNOS in both pulmonary artery and in the aorta as well as the expression of FP receptor in pulmonary artery.

The metabolic syndrome is related with different cardiovascular risk factors, including hypertension, TD2M, obesity and dyslipidemia [67]. TD2M is one of the main health problems worldwide and it is well known that is related with an augmented risk of microvascular and macrovascular problems, which include hypertension and atherosclerosis [21]. IR when associated with TD2M is indispensable for endothelial dysfunction [13], which is an important pathophysiological related with hypertension, obesity, diabetes and dyslipidaemia [15].

Effect of CSN resection on cardiometabolic parameters: insulin sensitivity, glucose tolerance and blood pressure

In this present work, we divided Wistar rats into two dietary groups, a standard diet and an HFHSu diet (60% of energy from fat) during twenty-five weeks. After fourteen weeks, some rats of both groups were submitted to a bilateral CSN resection. We have seen that the standard diet, even conjugated with the CSN resection, did not modify significantly insulin sensitivity or glucose tolerance. Nevertheless, as predictable, after fourteen weeks of HFHSu diet, the insulin sensitivity decreased and glucose intolerance augmented when compared with CTL values, and maintained at least for more eleven weeks (figure 4.1 and 4.2). These findings were also observed by Chaar *et al.*, (2016), even though being in another animal model, they demonstrated in 10 weeks old male mice C57BL/6J, submitted to HFHSu diet (13% carbohydrates, 20% protein and 60% lipids conjugated with 20% sucrose in the drinking water) during 8 weeks, that the animals increased body weight and augmented fasting glycaemia, hyperinsulinemia and exhibited severe glucose intolerance [68]. In the current study, we have shown that the group fed with HFHSu diet and after fourteen weeks submitted to a CSN resection, that CSN denervation restored for completely the IR and brought back, even not significantly, to CTL values the glucose tolerance. These results are in agreement with the previous findings of our laboratory, although not in the same diet model, that CSN resection restores insulin sensitivity and glucose tolerance. In fact, Sacramento *et al.*, (2017) divided 3 months old Wistar rats, into three groups: one was fed with the standard diet, another with high-fat diet (45% fat, 35% carbohydrate and 20% protein) and the third with high-sucrose diet (35% sucrose in drinking water) during 28 days and demonstrated that the animals submitted to a bilateral CSN resection, had a completely restoration of the insulin sensibility in both hypercaloric models and also show through the administration of glucose intravenously, that the bilateral CSN resection of the high-fat diet model, decreased the glucose intolerance [54]. Sacramento *et al.*, (2017) also demonstrated that the unilaterally resection of CSN is not effective on restoring insulin action and glucose metabolism, because exist a compensation from the other CB, contradicting the effect of resection [54].

We describe that the animals fed with HFHSu diet had a significantly increased in the MBP, in comparison with the CTL diet animals (figure 4.3). These results go in accordance with Bourgoin *et al.*, (2008), that conducted a study in six weeks old male Sprague-Dawley rats, divided randomly into two dietary groups for four weeks: the control group fed with a standard chow and the test group fed with a HFHSu diet (16.5% of energy from fat, 60.5% of energy as carbohydrate and 22.9% of energy

as protein). They demonstrated that the MBP was higher in the HFHSu-fed rats than in those fed with the standard chow. Even they hypothesized that the rise in the blood pressure could be due to the altered endothelial function and the insulin resistance induced by the diet, as previous data indicated that this complications precede hypertension in animal models, another factor could be the increased in the SNS induced by the fat feeding [69]. In the current work, we demonstrated that the CSN resection in HFHSu model decreased the MBP, and similar results were also shown by Ribeiro *et al.,* (2013) who demonstrated that 3 months old Wistar rats divided into two groups being one fed with high-fat diet (45% fat, 35% carbohydrate and 20% protein) and another fed with high-sucrose diet (35% sucrose in drinking water) during 28 days, that both diet models after submitted to a CSN resection normalized the increase in MBP induced by the hypercaloric diets [60]. We did not find any difference in the MBP from the animals fed with the standard diet or the controls submitted to CSN resection.

Effect of CSN resection on endothelial function

Cardiovascular diseases conjugated with DM alters the vascular responsiveness to several vasoconstrictors and vasodilators [70]. In the present work, we investigated the physiological function of the arteries by using a small vessel wire myograph, which allowed the vessels to respond to different pathophysiological stimuli [64]. For that we used higher K^+ (80mM), that allow the subsequent normalization of tensions, resulting this in a contractile response, that is usually considered as indicative of the smooth muscle contractile capability. The higher K^+ did not change significantly the contractile responses in the pulmonary artery, although it is observable that the CSN resection decreases slightly the contractile responses to K^+ (figure 4.4). Gurney *et al.,* (2009), described in Wistar rats injected with streptozotocin during 3-4 months, a parallel result in the maximum constriction with K^+ (110mM), in the pulmonary artery [71]. Likewise, in our results, the aorta, the HFHSu diet did not change the contractile response of K^+ in comparison with CTL values. Silan., (2008) also showed equivalent results, in 8 weeks old male Wistar albino rats injected with streptozotocin, that no differences was spotted in the maximal contractile responses of aorta to KCl (80mM) between control and diabetic [72]. From these results, we can suggest that diabetes did not alter the contractile responses to unspecific stimuli. Additionally, in the current study we demonstrated that the contractile response to 80mM K^+ was lower in the CSN resection groups, in aorta.

The arteries were then constricted with a agonist like $PGF_2\alpha$, that is a powerful vasoconstrictor [73]. In this study, we described that $PGF_2\alpha$ is a more potent vasoconstrictor of aorta rings than of the pulmonary artery (figure 4.5). We demonstrated that at the maximum $PGF_2\alpha$ concentration used (10μM), a difference exists between the animals fed with the HFHSu and those fed with the standard diet, and even among the animals submitted to CSN resection, in both arteries, having the pulmonary artery a decreased contractible response to $PGF_2\alpha$ in HFHSu animals. Our results were not consensual with the findings of Gurney *et al.,* (2009) that, described, in the pulmonary artery, that there was not statistical difference between the contractile response to $PGF_2\alpha$ in diabetic rats and the controls [71]. Probably these discrepancies, results from the fact that in the later study they induced diabetes in rats through an injection of streptozotocin and we induced diabetes by a HFHSu diet or even by the fact that they examined the arteries after 3-4 months and we in the present study after 25 weeks. On the other hand, we demonstrated in aorta that the HFHSu diet had a higher contractible response to $PGF_2\alpha$ compared with the CTL, and similar results were obtained by Miike *et al*., (2008) in 11 weeks old male *db/db* mice [74]. CSN resection did not changed significantly the contractile response to $PGF_2\alpha$ in both arteries.

It is known that diabetes and IR are associated to an impaired in the endothelium-dependent relaxation [23], and as previously describe above the ACh as the capacity to relax the VSMC indirectly by stimulating the release of NO, via increasing intracellular Ca^{2+} [15]. So, we evaluated the endothelial

integrity, by the use of concentration-response curves for relaxation to ACh, previously contracted with $PGF_2\alpha$ in the pulmonary artery and in the aorta (figure 4.6). In both arteries, as expectedly, the HFHSu diet displayed an impairment in the relaxation curves to ACh. Similar results were observed by Lopez-lopez *et al.,* (2008), which showed in pulmonary artery, an impairment in the relaxation to ACh, previous contracted with phenylephrine, in male Sprague-Dawley rats induced with diabetes by streptozotocin for six weeks [21]. Furthermore, Robert P. Hof & Akiko Hof., (1988) described in Mongrel rabbits, of 8-10 weeks of age, fed with 2% cholesterol, that the relaxant response to ACh, previous contracted with noradrenaline, was almost completely absent in the aorta [75]. This impaired endothelium-dependent relaxation in HFHSu could be due to hyperglycaemia, a decrease influx of Ca^{2+} into endothelium or due to a diminished release of Ca^{2+} from its intracellular stores, a reduced diffusion of NO into the VSCM [76] or due to endothelial damage resulting from increased ROS induced by DM [77]. One of the most remarkable results of our work is that herein we describe, for the first time that the CSN resection in the HFHSu animals has the capacity to improve the relaxation in both arteries, restoring almost completely the endothelial function. Also, the CSN resection did not altered the concentration-response curve for relaxation in the CTL animals.

Exploring the mechanisms beyond endothelial dysfunction and its restoration by CSN denervation

Multiple mechanisms can function simultaneously to induce endothelial dysfunction and therefore in the current study, we investigated two of the mechanisms that we believe to be essential to explain the reduced endothelium dependent vasodilation induced by the HFHSu diet. The consensual feature in endothelial dysfunction is a decrease in the bioavailability in the NO levels in the vasculature [15], so herein, we quantify the NO levels in plasma, in pulmonary artery and in the aorta (figure 4.7). We observed, as expected that in the plasma the NO levels were significantly higher in HFHSu groups. Our group has previously described increased NO plasma levels in hypercaloric models of metabolic syndrome [54,78]. In humans, Maejima *et al.,* (2001) also described in TD2M subjects with 59±13 years old, an increase in NO levels in plasma compared with the nondiabetic subjects [79]. As we previously showed in hyypercaloric models of metabolic syndrome, CSN resection decreased the NO levels in the plasma in both animals fed with the standard and HFHSu diet.

In the pulmonary artery, a non-significant decrease in the NO levels was found in the HFHSu group without CSN resection, being the reduction significant in the HFHSu-denervated group. Also, in the aorta, the HFHSu diet decreased significantly the NO levels compared to CTL values. Sena *et al*., (2008) also described in aorta of 15 months old spontaneously diabetic GK rats, a decreased in the NO levels when compared with the control values [47]. This reduction in NO levels could result in an increase in the vasoconstriction, which go in harmony what we describe above in the response constriction to $PGF_2\alpha$ in aorta of HFHSu animals, or due to an inflammation via an upregulation of leukocyte and vascular hypertrophy and stenosis on the vasculature [80]. Also, surprisingly, the CSN resection in the aorta in CTL animals increased significantly and the CSN resection in the HFHSu animals do not modify the NO levels (figure 4.8). These results indicate that the regulation of NO production by an HFHSu diet may be quite complex and several pathways may exist simultaneously.

In addition, we also explored in the present project, through Western Blot, the expression of the proteins eNOS, iNOS and FP receptor with the aiming to correlate the expression of proteins with the reduced vasodilation in the animal model HFHSu. Several studies have demonstrated that the common feature of endothelial dysfunction is a diminished eNOS expression, as described by Yang *et al.,* (2007) that displayed in male Sprague-Dawley rats fed with high-fat diet (49.85% fat, 20% protein and 30.15% carbohydrate) a lower levels of eNOS expression in aorta [81]. However, our results show that in the pulmonary artery and aorta, there is no significant changes in the eNOS expression, between the

CTL and HFHSu diet and the CSN resection animals (figure 4.9). Moral-Sanz *et al.*, (2011) also described in 17 weeks old obese Zucker rats (fa/fa), that eNOS expression levels were unchanged in the pulmonary artery [23], being these results also demonstrated by Lopez-lopez *et al.*, (2008), which alleged that eNOS deficiency in pulmonary artery might not account for endothelial dysfunction [21]. Furthermore, similar results were described in aorta of 6 months old Goto-Kakizaki rats by Carvalho (2011) [82].

In the literature is described that iNOS expression in the macrophages, is activated by inflammatory diseases, including diabetes and atherosclerosis [50], and therefore we decided to investigated the effect of HFHSu diet in the levels of expression iNOS and if the impact of CSN resection on these levels (figure 4.10). Herein, we observed that animals fed with HFHSu diet had an increased in the iNOS expression, in both arteries. Moral-Sanz *et al.*, (2011) also described, in 17 weeks old obese Zucker rats (fa/fa), an upregulation of iNOS expression levels in the pulmonary artery, and suggested that iNOS may be a source of the NO responsible for the vascular hyporesponsiveness in the rats [23]. Likewise, in the aorta, Nagareddy *et al.*, (2005) described in diabetic male Wistar rats induced with streptozotocin for 12 weeks, a higher expression of iNOS and hypothesize that the increased in the iNOS expression, could be direct involved with endothelial dysfunction [83]. Surprisingly, CSN resection in CTL animals in the pulmonary artery augmented the expression levels of iNOS. In contrast, CSN resection in the HFHSu animals decreased the expression of iNOS in both arteries, to similar values of the controls.

Additionally, we investigate the expression levels of the FP receptor to try to find an explanation for the differences in the contraction pattern, and we observed that the HFHSu diet increased the FP receptor levels of expression in the pulmonary artery, suggesting that this overexpression may contribute to the development and progression of vascular thickness and vascular remodelling in TD2M rats [84]. On the other hand, the HFHSu diet did not altered the FP receptor levels in the aorta (figure 4.11). Contrarily to our results, Li *et al*., (2015) described in male Sprague-Dawley, fed with a high-fat diet (34.5% fat, 15.5% protein, 48% carbohydrate) and injected with streptozotocin, an increased in FP receptor levels in aorta [84]. However, some differences in the animal model and the mean of inducing diabetes used can be in the basis of these discrepancies. In the aorta, surprisingly, the CSN resection decreased the expression levels of the FP receptor in CTL animals and did not modify the expression of FP receptor in HFHSu animals, an effect that was not surprising as the contraction in response to $PGF_2\alpha$ was not modified by CSN resection in the aorta. However, herein we demonstrated that HFHSu animals submitted to the CSN resection, exhibit a diminished expression in FP receptors levels in the pulmonary artery, nevertheless the contraction of the pulmonary artery in response to $PGF_2\alpha$ was not modified by CSN resection. Li *et al*., (2015) in the same study above described, silenced the FP receptor gene and found an attenuation in the vascular remodelling and considered that FP receptor could be an attractive drug for diabetic macrovascular complications [84].

We have found here several discrepancies between our results and those from other groups, but we believed that this could be due to different kinds of animals employed, the use of drugs or diet to induce diabetes, the duration of diabetes, age of animals and even different experiment protocols.

In the present thesis, we did not had enough time to explore more the mechanism behind the endothelial dysfunction linking the CB activity or between how the CSN resection can improve the endothelial function. So, to unveil these mechanisms, further experiments must be made. There are two pathways that we believe to be pertinent for the link between the CB and endothelial dysfunction: the inflammatory pathway and the SNS pathway or perhaps both, in simultaneously. It is well recognized that SNS plays a major role in the maintenance of homeostasis due to its participation in the cardiovascular system and in several metabolic pathways [58]. Also, it is known that an

overactivation of SNS is related with IR, obesity, hypertension, diabetes, and likewise is link to an increased inflammatory cytokine production [85,86,58]. Is still unknown if the SNS activation is associated with endothelial dysfunction in subjects with metabolic syndrome, however a common cardiovascular risk factors, such LDL cholesterol levels, are associated with reduced endothelial function and sympathetic activation [86]. Recently, Ribeiro *et al*., (2013) proposed that the CB is responsible by the development of IR and arterial hypertension through an overactivation of the sympathetic nerve activity, [60] and is known that the CBs participate in the regulation of blood pressure and cardiac performance through the SNS activation [58]. All these characteristics of metabolic diseases were reversed by the CSN resection [60], depriving the SNS activity [58]. Our results in this project, go along with describe above, where we saw that CSN resection may represent a putative target to reverse endothelial dysfunction associated with TD2M.

The other possible mechanism is through the hypercaloric diets that induce inflammation, which in turn is going to overactivate the CB and consequently increase the SNS activity and produce endothelial dysfunction [87]. The increased in inflammation has been recognized as a key point in the insulin resistance, diabetes, obesity, hypertension and hyperlipidemia [88,87], and it is seems involved in the endothelial dysfunction [89]. In inflammatory diseases, several mediators of the endothelial dysfunction are released, as the TNF-α, that when connected with is receptor decreases the eNOS protein expression and likewise induces the expression of intercellular adhesion molecules [89]. The reduction in the eNOS expression plus the eNOS uncoupling mediated by ROS, leads to a decreased in the NO production, consequently reducing vasodilation [89]. Therefore, to further access if inflammation is related with the results obtained in the levels of expression of iNOS in HFHSu animals, it is imperative to investigate the plasma biomarkers, including the intercellular adhesion molecules, like intercellular adhesion molecule 1, vascular cell adhesion molecule and E-selection. This molecules are normally expressed in the endothelial cell in response to the activation by inflammatory cytokines [89].

6. Conclusion

Vascular endothelial dysfunction is believed to be an initial step during atherosclerosis, hypertension, pulmonary hypertension and diabetes mellitus. Therefore, it is imperative to clarify the biological mechanisms underlying it and as well to find out strategies to prevent and treat.

This study demonstrated, for the first time, that carotid sinus nerve resection can restore the systemic endothelial dysfunction as well as the pulmonary endothelial dysfunction in a model of an early phase of type 2 diabetes, reversing the impaired relaxation to ACh and re-establish the normal levels of NO in plasma and the expression levels of iNOS in both arteries and $PGF_2\alpha$ R in the pulmonary artery. All together these results, suggest that the modulation of CB activity is important therapeutically for the treatment of hypertension and endothelial dysfunction related with type 2 diabetes.

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